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(54) Title: ANTISENSE OLIGONUCLEOTIDES AS ANTIBACTERIAL AGENTS

(57) Abstract

A novel method is provided that teaches the therapeutic use of nuclease resistant oligonucleotides for treating animals having an infection caused by a pathogenic bacterium. The method involves the integration of (1) methods for selecting the correct oligonucleotide, (2) synthesis and purification of nuclease resistant oligonucleotides, and (3) methods for *in vitro* analysis of potential antimicrobial oligonucleotides. The described oligonucleotides may comprise modified backbones, sugar residues, bases, or mixtures and have been subject to purification resulting in oligonucleotides that are capable of inhibiting the growth of a broad spectrum of clinically relevant bacterial species.

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ANTISENSE OLIGONUCLEOTIDES AS ANTIBACTERIAL AGENTS

The present application claims priority to United States Patent Application Serial No. 08/685,575, filed July 24, 1996.

FIELD OF THE INVENTION

The present invention is directed to methods for treating an animal, including a human, having a bacterial infection which comprise administering an oligonucleotide specifically targeted to, or otherwise capable of interacting with, a bacterial sequence, or nucleic acid binding protein. The antibacterial oligonucleotide inhibits the growth of the bacteria, blocks the expression of virulence factors or genes involved in the transfer of genetic information, or kills the bacteria. Alternatively, the oligonucleotide may also be targeted to an antibiotic resistance gene in order to render the bacteria sensitive to an otherwise ineffective antibiotic. The invention also relates to nuclease resistant oligonucleotides that are effective in inhibiting the growth of, or killing, pathogenic bacteria.

1.0. BACKGROUND TO THE INVENTION

1.1. Antibiotic Prior Art

Pathogenic bacteria responsible for infectious diseases were once thought to be totally under control through the use of a battery of antibiotics such as penicillin, streptomycin, tetracycline, and others. However, since the widespread use of antibiotics began in the 1950s, more and more bacteria resistant to one or more antibiotics have arisen. Multiple drug resistant strains are increasingly common, particularly in hospitals.

Currently, nosocomial Staphylococcal infections exhibit multiple drug resistance. See, for example, Archer *et al.*, Antimicrob. Agents Chemother. 38:2231-2237 (1994). At this time, the remaining antibiotic that demonstrates the ability to kill *Staphylococci* is vancomycin. Strains of *Enterococci* that are vancomycin resistant have already been isolated and

reported by Zabransky *et al.*, J. Clin. Microbiol. 33(4):791-793 (1995). Furthermore, transfer of resistance from *Enterococci* to *Staphylococci* has been previously documented by Woodford *et al.*, J. Antimicrob. Chemother. 35:179-184 (1995). *Streptococcus pneumoniae* is a leading cause of morbidity and mortality in the United States (M.M.W.R., Feb. 16, 1996, Vol. 45, No. RR-1). Each year these bacteria cause 3,000 cases of meningitis, 50,000 cases of bacteremia, 500,000 cases of pneumonia, and 7,000,000 cases of otitis media. Case fatality rates are greater than 40% for bacteremia and greater than 55% for meningitis, despite antibiotic therapy. In the past, *Streptococcus pneumoniae* were uniformly susceptible to antibiotics; however, antibiotic resistant strains have emerged and are becoming widespread in some communities.

In addition, there are instances where antibiotic resistance is not an issue, yet a particular bacteria remains refractory to treatment using conventional antibiotics. Such is the case with *Escherichia coli* 0157:H7, the causative agent for food poisoning and death from undercooked meat. The Department of Agriculture estimates that 10 people die each day and another 14,000 become ill due to this bacteria. Unfortunately, conventional antibiotics are completely ineffective against this organism.

The history of antibiotic treatment of pathogenic bacteria is cyclical. Bacteria are remarkably adaptive organisms, and, for each new antibiotic that has been developed, resistant bacterial strains arise through the widespread use of the antibiotic. Thus, there is a constant need to produce new antibiotics to combat the next generation of antibiotic resistant bacteria. Traditional methods of developing new antibiotics have slowed, and in the past two years only one new antibiotic has been approved by the FDA. Furthermore, according to Kristinsson (Microb. Drug Resistance 1(2):121 (1995)), "There are no new antimicrobial classes with activity against resistant Gram positives on the horizon."

1.2. Antisense Nucleotide Art

Antisense polynucleotides are useful for specifically inhibiting unwanted gene expression in mammalian cells. They can be used to hybridize to and inhibit the function of an RNA, typically a messenger RNA, by activating RNase H or physically blocking the binding of ribosomes or proteins, thus preventing translation of the mRNA. Antisense oligonucleotides also include RNAs with catalytic activity (ribozymes), which can selectively bind to complementary sequences on a target RNA and physically destroy the target by mediating a cleavage reaction.

Antisense oligonucleotides that bind to the DNA at the correct location can also prevent the DNA from being transcribed into RNA. These antigene oligonucleotides are believed to bind to double-stranded DNA (forming triple-stranded DNA) and thereby inhibit gene expression.

1.3. Antisense Nucleotides For Therapy

The use of antisense oligonucleotides has emerged as a powerful new approach for the treatment of certain diseases. However, the preponderance of the work to date has focused on the use of antisense oligonucleotides as antiviral agents or as anticancer agents (Wickstrom, E., Ed., Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS, New York: Wiley-Liss, 1991; Crooke, S.T. and Lebleu, B., Eds., Antisense Research and Applications, Boca Raton: CRC Press, 1993, pp. 154-182; Baserga, R. and Denhardt, D.T., 1992, Antisense Strategies, New York: The New York Academy of Sciences, Vol. 660; Murray, J.A.H., Ed., Antisense RNA and DNA, New York: Wiley-Liss, 1993).

There have been numerous disclosures of the use of antisense oligonucleotides as antiviral agents. For example, Agrawal *et al.* report phosphoramidate and phosphorothioate oligonucleotides as antisense inhibitors of HIV (Agrawal *et al.*, Proc. Natl. Acad. Sci. USA 85:7079-7083 (1988)). Zamecnik *et al.* disclose antisense oligonucleotides as inhibitors of Rous sarcoma virus replication in chicken

fibroblasts (Zamecnik *et al.*, Proc. Natl. Acad. Sci. USA 83:4143-4146 (1986)).

There seem to be few to no toxicity problems associated with the use of antisense oligonucleotides as drugs to treat disease. To date, no dose limiting toxicities of phosphorothioate antisense oligonucleotides have been detected in man (Crooke, S.T., "Progress in Oligonucleotide Therapeutics," Abstracts American Association for Cancer Research, March 18-22, 1995; Crooke, S.T., "Progress in Oligonucleotide Therapeutics," Abstracts Oligonucleotide-Based Therapeutics, February 9-10, 1995), and phosphorothioate oligonucleotides have been found to have no effect on developing embryos (Guadette *et al.*, Antisense Res. Devel. 3:391-397 (1993)). In fact, under an emergency IND approval, a 19-year-old male received 700 mg of an antisense phosphorothioate oligonucleotide to treat acute myeloblastic leukemia (Bayever *et al.*, Antisense Res. Devel. 2:109-110 (1992)). There were no changes in pulse, respiratory rate, blood pressure, fever, mucositis, or diarrhea in the patient. In addition, no neurological, cardiovascular, respiratory, renal, skin or nephrourological toxicities were observed. It was concluded that systemic administration of a phosphorothioate antisense oligonucleotide to humans achieves adequate bioavailability of the drug to target tissues without major toxicity. In a follow up study, the antisense phosphorothioate oligonucleotides were given to five patients with acute myeloblastic leukemia. After systemic intravenous administration of the oligonucleotide, no toxic effects were seen. See Fig. 1 of Bayever *et al.*, Antisense Res. Devel. 3:383-390 (1993). The authors concluded that the favorable pharmacokinetics observed support the use of phosphorothioate oligonucleotides as potential gene specific therapeutic agents.

1.4. The Transport Problem For Oligonucleotides

While the use of antisense oligonucleotides as antiviral agents has been described (Agrawal *et al.*, Pat. No. 5,194,428, issued March 16, 1993), no significant progress has been made in the therapeutic use of antisense oligonucleotides to treat bacterial infection. In fact, at a recent meeting on Antibiotic Discovery addressing the current state of the art, there were no talks or discussions scheduled regarding the use of antisense oligonucleotides to treat bacterial infections, although the use of antisense oligonucleotides as antiviral agents was scheduled for discussion ("Antibiotic Discovery," Abstracts International Business Communications, June 26-27, 1995).

Logically, the use of synthetic oligonucleotides should be advantageous as an approach to treating bacterial infection because sequences can be specifically designed to inhibit bacterial growth while not interfering with the metabolism of mammalian cells.

In addition, oligonucleotides have been shown to nonspecifically stimulate the immune system (Yamamoto *et al.*, Antisense Res. Devel. 4:119-122 (1994); Krieg *et al.*, Nature 374:546-549 (1995)). Since current antibiotics generally function by arresting bacterial growth until the immune system can respond to the infection (Myrvik, Fundamentals of Medical Bacteriology, 1974, Lea & Febiger, Publishers), the use of oligonucleotides as antibiotics may provide both a nonspecific stimulation of the immune system as well as the relatively specific inhibition of the growth of a particular bacteria.

Furthermore, infectious bacteria have been shown to become sequestered in the liver and spleen in clinical infections (Wilson, G.S. and Miles, A.A., Eds., Topley and Wilson's Principles of Bacteriology and Immunology, Williams & Wilkins, Publishers, 1964). Oligonucleotides, or more specifically S-oligonucleotides (phosphorothioate substituted), have also been shown to accumulate in these organs (Agrawal *et al.*, Proc. Natl. Acad. Sci. USA 88:7595-

7599 (1991)). Therefore, the use of antisense oligonucleotides should be ideally suited to the treatment of bacterial infections involving the liver and spleen as well as systemic bacteremia and septicemia.

The rigid cellular architecture of the prokaryote has been viewed as a barrier to oligonucleotide uptake by bacterial cells (Chrisey *et al.*, Antisense Res. Devel. 3:367-381 (1993)). In fact, reports of antisense oligonucleotide-mediated gene inhibition in bacteria have attempted to circumvent the perceived problem of the rigid cell wall by conducting experiments in cell-wall deficient strains (Jayaraman *et al.*, Proc. Natl. Acad. Sci. USA 78:1537-1541 (1981)), in competent bacterial cells (Ciferri *et al.*, J. Bacteriol. 104:684-688 (1970)), in heat-shock permeabilized bacteria (Gasparro *et al.*, Antisense Res. Devel. 1:117-140 (1991)), in hypertonic solutions (Chrisey *et al.*, Antisense Res. Devel. 3:367-381 (1993)), and using PEG-modified oligonucleotides (Rahman *et al.*, Antisense Res. Devel. 1:319-327 (1991)), none of which has relevance to treating clinical bacterial infections.

Lupski *et al.*, Pat. No. 5,294,533 ('533 patent), stated that antisense oligonucleotides can preferentially inhibit the growth of Gram negative and Gram positive bacteria in a mixed culture of Gram negative and Gram positive bacteria. Lupski *et al.* also taught that end-capped oligonucleotides should be used (see column 4, lines 39-42), but since end-capping does not provide protection from intracellular endonucleases (see the discussion of Hoke *et al.* above), one skilled in the art would not expect the method of Lupski *et al.* to work. Thus, the '533 patent does not provide an enabling description of the use of antisense oligonucleotides to inhibit the growth of bacteria *in vivo* in mammals.

Moreover, the '533 patent did not disclose the genotype of the bacteria used in the study. Thus, there is no way to establish whether clinical isolates were used or permeability enhanced bacterial mutants were used. Additionally, the '533 patent does not provide adequate teaching to allow one to

discern whether or not the described bacteria had been previously rendered competent by established prior art methods. In view of this lack of disclosure, the '533 patent does not teach methods that are broadly applicable to clinically significant bacterial infections in mammals.

The prior art teaches the inherent difficulty of successfully using oligonucleotides to inhibit the growth of intact bacteria (Jayaraman *et al.* and Ciferri *et al.*), and the '533 patent does not provide sufficient disclosure to refute the clear teaching in the prior art. Instead, the '533 patent simply states that: "A small 10-29 mer antisense oligonucleotide that is delivered to a bacteria is rapidly transported into the bacterial cells." This statement is clearly contrary to what is taught by the prior art.

The prior art has never conclusively established that the growth of wild type bacteria may be inhibited by either nuclease resistant or nuclease sensitive oligonucleotides. It was also well known that methylcarbamate modified oligonucleotides (the methylcarbamate replaced the phosphodiester bonds) of three and four nucleotide units, and methylphosphonates longer than four nucleotide units could not enter *Escherichia coli* cells (Jayaraman *et al.*, Proc. Natl. Acad. Sci. USA 78:1537-1541 (1981), Rahman *et al.*, Antisense Res. Devel. 1:319-327 (1991)). Thus, the prior art teaches that the alleged results described in the '533 patent conflict with previously reported results from bacterial experiments using nuclease resistant oligonucleotides, or phosphodiester oligonucleotides.

In 1993, Chrisey reported uptake *in vitro* of phosphorothioate oligonucleotides into *Vibrio* bacteria under hypertonic conditions, and were only able to show uptake when the cells were grown under conditions that enhanced the permeability of the bacterial cells (*i.e.*, in a hypertonic minimal medium). From these data, Chrisey *et al.* concluded that, in enriched media (blood, serum, and other extracellular fluids), oligonucleotides may not be preferred antibacterial agents for use *in vivo*.

1.5. Oligonucleotides As Antibacterial Agents

As discussed above, essentially five publications have addressed the possibility of using oligonucleotides to inhibit bacterial growth. Four out of five of these publications (Rahman, Chrisey, Jayaraman, and Gasparro) teach that oligonucleotides are not able to inhibit the growth of unmodified (intact) bacteria. Additionally, the last reference (Lupski) provides no teaching of how to inhibit the growth of intact bacteria, and provides no illustrative examples that such inhibition is indeed possible.

Taken as a whole, the above publications would have not provided a reasonable expectation that one could in fact use oligonucleotides to inhibit the growth of intact bacteria. The inadequacies of the background art may be explained by the fact that the present applicants have discovered that at least several features of the design, preparation, and use of oligonucleotides may affect antibacterial efficacy. These features include, but are not limited to: 1) the dose of oligonucleotide; 2) the length of the oligonucleotide; 3) the growth conditions used during the *in vitro* assay; 4) the chemical backbone of the oligonucleotide; and 5) the method of post-synthesis purification. Each of these features are discussed in greater detail below.

The dose of oligonucleotide may significantly effect the observed amount of growth inhibition. Fig. 1 shows that the percent of inhibition varies from 100% down to about 19% as the dose of oligonucleotide is reduced from 285 μM to 5 μM in a standard MIC assay (described in Section 4.5, infra). Of the background references, only Rahman and Jayaraman taught concentrations of oligonucleotide that fall within the disclosed range (but observed little to no inhibitory effect against intact bacteria).

The applicants have also found that the length of the oligonucleotide is directly related to its ability to specifically bind and inhibit the normal function of the target sequence. Shorter oligonucleotide sequences generally have a reduced Tm (duplex melting temperature) and are thus

more likely to cause undesirable side effects of nonspecific binding or have no effect. Gao *et al.*, Molec. Pharm. 41:223-229 (1992) have shown that, using an *in vitro* enzymatic assay, the inhibitory effect of an oligonucleotide sequence increased as the length of the oligonucleotide was progressively increased from a 7mer up to a 28mer. Gao *et al.* observed no specific inhibitory activity when a 7mer was tested. Of the cited references, Rahman, Jayaraman, Gasparro, and Chrisey used oligonucleotides that were a maximum of only 12 bases in length. Typically, oligonucleotides as short as the disclosed 12mers show a high degree of nonspecific binding. Lupski chose sequences of about 25 bases in length but the majority of the disclosed sequences comprised a high degree of degeneracy which allows for binding to multiple target sites. For example, oligonucleotides comprising bases such as inosine, or "N" (which indicates the use of A, C, G, or T), are usually produced when one wishes to allow binding to sequences where the precise target sequence is unknown (Ohtsuka et al., J. Biol. Chem. 260:2605 (1985)). Sequences with such broad based homology run the risk of nonspecific binding to host sequences and associated toxicity effects. Additionally, Lupski's teaching is inherently suspect given that no data demonstrating the inhibition of bacterial growth was provided.

It should also be noted that shorter oligonucleotide sequences generally have reduced Tm's. The oligonucleotides taught by Rahman, Jayaraman, Gasparro, and Chrisey were generally so short that the Tm's for the oligonucleotide-target sequence hybrids were usually below 37° C. For example, the 12mer phosphorothioate sequence taught by Chrisey has a predicted Tm of 28.9° C, the 9mer taught by Gasparro had a predicted Tm of 24.7° C, and the 7mer (AGGAGGT) taught by Jayaraman and 4mer (GGAG) taught by Rahman both had a predicted Tm's well below 10° C. Given these data, it is clear that oligonucleotides of the length

taught by these references are generally not useful as antisense or antigene agents under physiologic conditions.

The growth rate and conditions under which antibiotic susceptibility are measured may profoundly effect a bacterium's sensitivity to antibacterial agents (Arrow *et al.*, Antimicrob. Agents Chemother. 26:507 (1984)), and the uptake of the antibiotic into the cell (Arrow *et al.*, Microbiol. Rev. 51:439-457 (1987)). Accordingly, methods for screening oligonucleotides *in vitro* for antibacterial activity should generally be conducted under standardized conditions that reflect the *in vivo* circumstances of a given pathogen such as the NCCLS MIC tests (see Section 4.5, *infra*). None of the background references recognized that growth conditions might effect the result of antibiotic susceptibility tests, and thus none of these references assayed for the inhibition of bacterial growth using the standardized growth conditions defined in the MIC tests.

Among other things, the antibacterial efficacy of an oligonucleotide may be directly related to the relative nuclease resistance of the chemical backbone of the oligonucleotide. Gasparro and Lupski did not recognize this facet of the present invention and thus did not teach oligonucleotides that were designed to be nuclease resistant. Consequently, the oligonucleotides used by Gasparro and Lupski would have been rapidly degraded by the cell (see Section 1.6, *infra*), and would thus have little utility as antibacterial agents.

Additionally, the post-synthesis handling and purification of the oligonucleotides may profoundly effect antibacterial efficacy. None of the background references recognized the particular importance of post-synthesis handling, and thus none of the references explicitly suggest or describe purification protocols that produce effective antibacterial oligonucleotides.

In summary, none of the background references recognized the importance of the features described above. In brief, Rahman and Jayaraman both failed to provide explicit teaching

of oligonucleotides of the correct length, the use of proper susceptibility assays, or the correct purification scheme; Gasparro failed to explicitly teach the correct dose of oligonucleotide, oligonucleotides of the correct length, the use of proper susceptibility assays, the importance of nuclease resistant backbones, or the correct purification scheme; Chrisey failed to explicitly teach the correct dose of oligonucleotide, oligonucleotides of the correct length, the use of proper susceptibility assays, or the correct purification scheme; and Lupski failed to explicitly teach the correct dose of oligonucleotide, the use of proper susceptibility assays, the importance of nuclease resistant backbones, or the use of purified oligonucleotides. The background references, considered as a whole, failed to recognize the importance of all of the features described above. Furthermore, none of the background references used intact clinical isolates for their studies. Accordingly, the use of oligonucleotides to inhibit the growth of clinically relevant (*i.e.*, intact) strains of bacteria remained elusive. Conversely, the present disclosure teaches the importance of all of the above features, and integrates all of them to provide the first teaching of the use of antibacterial oligonucleotides to inhibit the growth of clinically relevant bacterial pathogens.

1.6. Nuclease Resistant Oligonucleotides

It has been demonstrated that the fate of internalized oligonucleotides is critical to the success of antisense gene therapy (Bennett, Antisense Res. Devel. 3:235-241 (1993)). The rapid intracellular degradation of oligonucleotides is a barrier to efficient inhibition of gene expression. One of the major problems in utilizing naturally occurring phosphodiester oligonucleotides is their rapid degradation by nucleases in mammalian cells or in serum-containing culture medium (Cohen, Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression, Boca Raton, Fla., CRC Press (1989)). There is abundant evidence that modification of the backbone

of oligonucleotides confers varying degrees of nuclease resistance. Hoke *et al.*, Nucl. Acids Res. 19:5743 (1991) compared phosphodiester backbone oligonucleotides to fully modified phosphorothioate backbone oligonucleotides, and to chimeric phosphodiester and phosphorothioate backbone oligonucleotides. Hoke *et al.* demonstrated that the phosphorothioate oligonucleotides were degraded up to 45 times slower than the phosphodiester or chimeric backbone oligonucleotides.

There have been reports that chimeric oligonucleotides that are end-capped with nuclease resistant backbone linkages are resistant to degradation (Cohen, "Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression," Boca Raton, Fla., CRC Press (1989)). However, Hoke *et al.* teach that capped oligonucleotides are rapidly degraded by intracellular endonucleases, and thus, that capping oligonucleotides with nuclease resistant modifications may not be sufficient for sustaining pharmacological activities of oligonucleotides in cells. Finally, Hoke *et al.* concludes that while capping of oligonucleotides may provide protection from exonucleases in cell culture, the action of intracellular endonucleases is sufficient to degrade these capped oligonucleotides when they enter a cell.

Hoke *et al.* is corroborated by Gao *et al.* who studied the relationship between the structure of the phosphodiester/phosphorothioate chimeras and nuclease resistance. Gao *et al.* showed a correlation between the number of phosphorothioate linkages and nuclease resistance of the oligonucleotide.

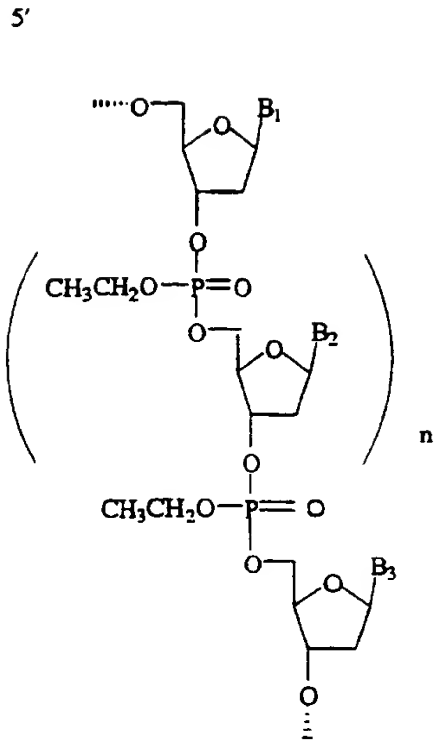
Extending these concepts, Zhao *et al.*, Antisense Res. Devel. 3:53-66 (1993), have looked at the effects of backbone modifications on cellular uptake of oligonucleotides in eukaryotes. This is an important property as the efficacy of an antisense oligonucleotide will be influenced by cellular uptake. Zhao *et al.* demonstrated that cell surface binding and uptake was greatest for phosphorothioate oligonucleotides followed by phosphodiester/phosphorothioate chimeras, and

finally by phosphodiester backbone oligonucleotides. Chrisey *et al.*, Antisense Res. Devel. 3:367-381 (1993), looked at the uptake and stability of phosphodiester and phosphorothioate backbone oligonucleotides by bacteria under hypertonic conditions. Chrisey *et al.* concluded that phosphorothioate 6mers were relatively resistant to nuclease activity in *Vibrio parahaemolyticus* cells and were relatively non-toxic. However, Chrisey *et al.* did not demonstrate that the internalized 6mers had antimicrobial activity.

Various modifications to the oligonucleotide backbone have been found to inhibit nuclease degradation. Such nuclease resistant modified nucleotides are well described in the literature and include, but are not limited to, the methylphosphonates, p-ethoxy deoxyribonucleotides, p-ethoxy 2'-O-methyl ribonucleotides, 2'-O-methyl ribonucleotides, phosphorothioates, and others. A brief description of representative nuclease resistant oligonucleotide backbones follows:

Methylphosphonate oligonucleotides, in addition to exhibiting enhanced nuclease resistance, also have increased hydrophobicity over phosphodiester oligonucleotides and therefore have greater permeability to cell membranes as compared to phosphodiester or other more highly charged oligonucleotides.

p-Ethoxy deoxyribonucleotides have an ethyl group o-linked to the phosphate backbone. p-Ethoxy deoxyribonucleotides are resistant to nuclease degradation. p-Ethoxy ribonucleotides have the following structure:

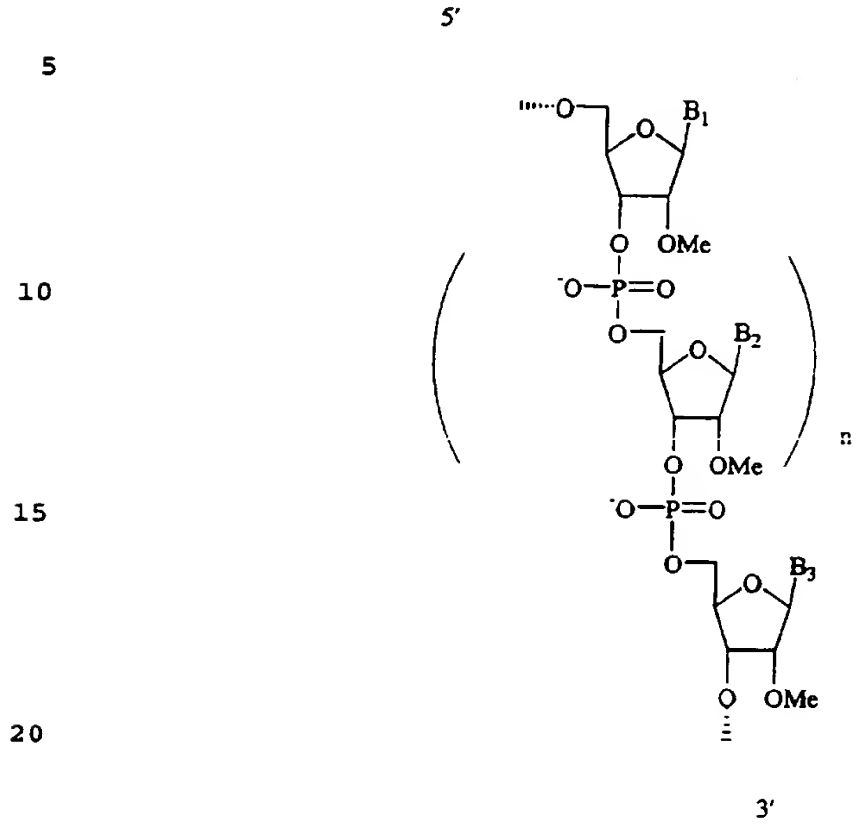


Phosphorothioates are compounds in which one of the non-bridging oxygen atoms in the phosphate backbone of the nucleotide is replaced by a sulfur atom. The phosphorothioates are resistant to cleavage by nucleases and, since they have the same number of charged groups as phosphodiester oligonucleotides, have good solubility in water. These compounds also exhibit more efficient hybridization with complementary DNA sequences than the corresponding methylphosphonate analogues.

Methyl carbonates are compounds in which one of the nonbridging oxygen atoms in the phosphate backbone has been replaced by a methyl carbonate group.

2'-O-methyl ribonucleotides are compounds in which the 2' position of the ribose sugar ring has a methoxy group in

place of the normal hydroxyl group. 2'-O-methyl ribonucleotides have the following general structure:



Secondary structure can also be used to make oligonucleotides resistant to nucleases. Oligonucleotides with a hairpin loop structure extending from the 3'-terminus, stabilizing the oligonucleotide against 3'-nucleolytic degradation, have been reported by Khan and Coulson, Nucl. Acids Res. 21(12):2957-2958 (1993). The Tm of the modified oligonucleotide from its complementary mRNA target was unaffected by the presence of the loop modification.

Further, end modification of oligonucleotides can also render an oligonucleotide resistant to nucleases, such as, for example, attaching cholesterol, psoralen, rhodamine, fluorescein, DNP, amine groups, biotin, inverted (3'-3' or 5'-5') linkages, and the like, to the end of the oligonucleotide in order to render it more nuclease resistant.

2.0. SUMMARY OF THE INVENTION

The present invention relates to methods for the treatment of animals, including humans, that have a bacterial disease. The preferred method of treatment comprises the administration of a purified antibacterial oligonucleotide having about 8 to about 80 nucleotides to the animal in an amount sufficient to inhibit bacterial growth, alleviate a symptom of the infection, or in an amount effective for treatment.

The purified antibacterial oligonucleotides of the present invention will preferably bear an enhanced ability to inhibit the growth of bacterial cells relative to previously disclosed oligonucleotide preparations. The present invention also represents the first disclosure of the use of oligonucleotides to inhibit the growth of intact clinically relevant bacteria. The oligonucleotides generally inhibit bacterial growth by acting as antisense or antigene inhibitors of bacterial gene expression (when targeted to bacterial nucleic acid sequences), or by acting aptamerically to alter the function of specific bacterial proteins or polypeptides (when associating target amino acid sequences contained in bacterial peptides, polypeptides, and proteins). Alternatively, the oligonucleotides are targeted to an antibiotic resistance gene to render the bacteria sensitive to a conventional antibiotic. In preferred embodiments, the antibacterial oligonucleotides are substantially nuclease resistant (*i.e.*, resistant to nuclease activity).

Additional embodiments of the present invention are antibacterial oligonucleotides that have been produced by a process that enhances the oligonucleotide's antibacterial activity. In particular, the presently described antibacterial oligonucleotides will be produced, or otherwise purified, by a process comprising either individually or in combination ion exchange or reverse phase chromatography, extractions, precipitations, gel filtrations, dialysis, diafiltration or functional equivalents. Column chromatography may be by traditional of methods or High-

Performance Liquid Chromatography (HPLC), fast performance liquid chromatography (FPLC), and the like. Additionally, the oligonucleotides may be purified by processes including, for example, extraction or precipitation with alcohols or organic solvents.

The present invention further contemplates the use of the described antibacterial oligonucleotides, in conjunction with an acceptable pharmaceutical carrier, to prepare medicinal compositions for the treatment of bacterial infections in animals, and more preferably mammals, including humans.

3.0. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a dose response curve of different concentrations of antibacterial oligonucleotide NBT 89 (SEQ ID NO. 61) when tested against *Escherichia coli* ATCC accession No. 25922.

Figure 2 provides a nonexhaustive graph of the types of bacterial genes which proved susceptible to inhibition by antibacterial oligonucleotides. The ordinate shows the categories of bacterial genes defined in Table 2(A-W).

Figures 3(a-c) show the percent inhibition of the growth of the indicated target bacteria after addition of the indicated oligonucleotide as a function of time.

Figures 4(a-c) show the percent inhibition of the growth of the indicated target bacteria after addition of the indicated oligonucleotide as a function of time.

Figures 5(a and b) show the percent inhibition of the growth of the indicated target bacteria after addition of the indicated oligonucleotide as a function of time.

Figures 6(a-t) are plots of log bacterial growth (and accompanying control cultures) as a function of time after the addition of the indicated oligonucleotide (*i.e.*, "NBT 114" indicates oligonucleotide sequence 114 (SEQ ID NO. 112) from Table 1, *infra*). A clinical isolate of *Escherichia coli* ATCC accession No. 35218 (multiple drug resistant) was used in the experiments corresponding to figures 6(a-t).

Figures 7(a-j) are plots of log bacterial growth (and accompanying control cultures) of the penicillin resistant clinical isolate of *Staphylococcus aureus* ATCC accession No. 13301 as a function of time after the addition of the indicated oligonucleotide.

Figures 8 shows that animals challenged with the bacterial pathogen *Escherichia coli* show a significant increase in survival after treatment with oligonucleotide 114 (SEQ ID NO. 112) relative to nontreated control animals.

Figure 9 shows that test animals infected with the bacterial pathogen *Staphylococcus aureus* show a significant increase in survival after treatment with the variant of oligonucleotide 114 (SEQ ID NO. 112), SOT 114.21, relative to nontreated control animals.

Figures 10(a-b) show the results observed when the indicated antibacterial oligonucleotides were tested for bactericidal activity against *Staphylcoccus aureus* using a standard overnight MIC assay.

Figures 11(a-b) show the results observed when the indicated antibacterial oligonucleotides were tested for bactericidal activity against *Serratia liquefaciens* using a standard overnight MIC assay.

Figure 12 shows the results obtained when the indicated antibacterial oligonucleotides were tested using a standard MIC assay against *Staph. aureus*.

Figure 13 shows the results obtained when a variety of different length versions of the indicated antibacterial oligonucleotide were tested using a standard MIC assay against *Staph. aureus*.

Figure 14 shows the results obtained when drug sensitive and drug resistant *Staph. aureus* were treated with oligonucleotide 114, and ampicillin.

Figure 15 shows the results of a standard MIC assay using oligonucleotide MMT 114.15 against *P. aeroginosa* strain 10145.

Figure 16 shows the results of a standard MIC assay using SOT 114.21 against *Strep. pyogenes* strain 14289.

4.0. DETAILED DESCRIPTION OF THE INVENTION

Prior to the present invention, clinically relevant bacterial pathogens were largely immune from treatment with antisense oligonucleotides. The reasons that the prior art oligonucleotides were ineffective against these pathogens include the dosages used, the lack of nuclease resistance of the oligonucleotide or the choice of the backbone, the length of the oligonucleotide, and the method of purification.

The present invention describes a method for generating oligonucleotides having the novel property of being capable of having bacteriostatic or bactericidal effects on clinically relevant bacterial pathogens. The oligonucleotides generated using the presently described methods are contemplated to be able to exert antibacterial effect both *in vitro* and *in vivo*. Typically, the antibacterial oligonucleotides will be targeted to bacterial sequences where, after associating with or binding to the target sequence, the oligonucleotide disrupts the normal function of the target sequence. The antibacterial effect of the oligonucleotide may be caused by either specific or nonspecific association as long as bacterial growth is inhibited.

Accordingly, particularly preferred embodiments of the present invention include the novel antibacterial oligonucleotides, methods of making the antibacterial oligonucleotides, and methods of using the novel antibacterial oligonucleotides to treat bacterial infection.

Given that bacterial infection is a particularly problematic complication in immunocompromised individuals such as patients suffering from acquired immunodeficiency disease syndrome (AIDS), HIV infected individuals, patients undergoing chemotherapy or radiation therapy, etc., an additional embodiment of the presently described invention is the use of the presently described antibacterial oligonucleotides to treat immunocompromised patients.

In a particularly preferred embodiment, the antibacterial oligonucleotides may be used to treat bacterial

infections in conjunction with similarly engineered antiviral oligonucleotides that are directed to any of a wide variety of human viruses including, but not limited to, adenovirus, human immunodeficiency virus, human leukemia virus, rhino virus, herpes virus, human papilloma virus, respiratory syncytial virus, cytomegalo virus, Epstein bar virus, hepatitis virus (A, B, C and delta), etc. Accordingly, an additional embodiment of the presently described invention are mixed oligonucleotide compositions that comprise both antiviral and antimicrobial (e.g., antifungal, antibacterial, antiparasitic, etc.) oligonucleotides. Preferably, the relative ratios of the oligonucleotides present in such compositions shall be adjusted to target bacterial, parasitic, fungal, yeast, and viral pathogens that are generally associated as secondary infectious sequelae of infection by one another.

An additional embodiment of the present invention are therapeutic oligonucleotides that fuse one or more sequences with known antimicrobial, antibacterial, or antiviral therapeutic activity. Such fusions are deemed to constitute novel compositions having broad spectrum activity against multiple and distinct bacterial species, as well as broad antiviral and antibacterial activities. Similarly, oligonucleotides bearing multiple active sequences, or mixed compositions of antibacterial oligonucleotides, may be used to target the activity of a gene product in an pathogen by blanket targeting of the DNA (via triplex inhibition, disrupting DNA replication, etc.) and RNA (via RNase H activation or directly disrupting translation, etc.) encoding the activity of interest, as well as by aptameric inhibition of the gene product.

Where the therapeutic use of the presently described antibacterial oligonucleotides is contemplated, the antibacterial oligonucleotides are preferably administered in a pharmaceutically acceptable carrier, via oral, intranasal, rectal, topical, intraperitoneal, intravenous, intramuscular, subcutaneous, intracranial, subdermal, transdermal,

intrathecal methods, or the like. Typically, the preferred formulation for a given antibacterial oligonucleotide is dependent on the location of the target organism in the host animal or the location in a host where a given infectious organism would be expected to initially invade.

For example, topical infections are preferably treated or prevented by formulations designed for topical application, whereas systemic infections are preferably treated or prevented by administration of compositions formulated for parenteral administration. Additionally, pulmonary infections may be treated both parenterally and by direct application of the antibacterial oligonucleotides to the lung by inhalation therapy.

Additionally, as oligonucleotides are cleared from the bloodstream, they can often accumulate at relatively high levels in the kidneys, liver, spleen, lymph glands, adrenal gland, aorta, pancreas, bone marrow, heart, and salivary glands. Oligonucleotides also tend to accumulate to a lesser extent in skeletal muscle, bladder, stomach, esophagus, duodenum, fat, and trachea. Lower still concentrations are typically found in the cerebral cortex, brain stem, cerebellum, spinal cord, cartilage, skin, thyroid, and prostate (see generally Crooke, 1993, *Antisense Research and Applications*, CRC Press, Boca Raton, FL). Interestingly, pathogenic bacteria also tend to accumulate in many of the above organs. Consequently, the presently described antibacterial oligonucleotides can be used to target bacterial infections in specific target organs and tissues.

One of ordinary skill will appreciate that, from a medical practitioner's or patient's perspective, virtually any alleviation or prevention of an undesirable symptom (*e.g.*, symptoms related to the presence of bacteria in the body) would be desirable. Thus, the terms "treatment", "therapeutic use", or "medicinal use" used herein shall refer to any and all uses of the claimed antibacterial oligonucleotides which remedy a disease state or symptoms, or otherwise prevent, hinder, retard, or reverse the progression

of disease or other undesirable symptoms in any way whatsoever.

Preferably, animal hosts that may be treated using the oligonucleotides of the present invention include, but are not limited to, invertebrates, vertebrates, birds (such as chickens and turkeys, etc.) fish, mammals such as pigs, goats, sheep, cows, dogs, cats, and particularly humans.

When used in the therapeutic treatment of disease, an appropriate dosage of an antibacterial oligonucleotide, or mixture thereof, may be determined by any of several well established methodologies. For instance, animal studies are commonly used to determine the maximal tolerable dose, or MTD, of bioactive agent per kilogram weight. In general, at least one of the animal species tested is mammalian. Those skilled in the art regularly extrapolate doses for efficacy and avoiding toxicity to other species, including human. Before human studies of efficacy are undertaken, Phase I clinical studies in normal subjects help establish safe doses. Additionally, therapeutic dosages may also be altered depending upon factors such as the severity of infection, and the size or species of the host.

The presently described antibacterial oligonucleotides may also be complexed with molecules that enhance their ability to enter the target cells. Examples of such molecules include, but are not limited to, carbohydrates, polyamines, amino acids, peptides, lipids, and molecules vital to bacterial growth.

Additionally, the antibacterial oligonucleotide may be complexed with a variety of well established compounds or structures that, for instance, further enhance the *in vivo* stability of the oligonucleotide, or otherwise enhance its pharmacological properties (*e.g.*, increase *in vivo* half-life, reduce toxicity, etc.).

The use of synthetic oligonucleotides are advantageous as an approach to treating bacterial infection because sequences can be specifically designed to inhibit bacterial

growth while not interfering with the metabolism of mammalian cells.

The present invention also relates to oligonucleotides that have demonstrated antibacterial activity *in vitro*. In particular, the oligonucleotides will have antibacterial activity as measured in a MIC (minimal inhibitory concentration) test that is recognized in the art as predictive of *in vivo* efficacy for the treatment of a bacterial infection with antibiotics. Without pretreatment of the bacteria to permeabilize them and without PEG-modification of the oligonucleotides, the oligonucleotides of the present invention are able to hybridize to a targeted region of a chosen bacterial polynucleotide (DNA or RNA) to effectively inhibit the ability of that polynucleotide to serve as a template for synthesis of its encoded product (DNA, RNA or protein), or otherwise inhibit the target sequence's normal function in the bacterium, thereby causing a bacteriostatic or bactericidal effect. Certain oligonucleotides may exert their bacteriostatic or bactericidal effects through binding to and inhibition of protein (aptameric effects).

In a preferred embodiment, the invention uses oligonucleotides that are substantially nuclease resistant. This includes oligonucleotides completely derivatized by phosphorothioate linkages, 2'-O-methylphosphodiesters, p-ethoxy oligonucleotides, p-isopropyl oligonucleotides, phosphoramidates, chimeric linkages, and any other backbone modifications, as well as other modifications, which render the oligonucleotides substantially resistant to endogenous nuclease activity. Additional methods of rendering an oligonucleotide nuclease resistant include, but are not limited to, covalently modifying the purine or pyrimidine bases that comprise the oligonucleotide. For example, bases may be methylated, hydroxymethylated, or otherwise substituted (glycosylated) such that the oligonucleotides comprising the modified bases are rendered substantially nuclease resistant.

The present invention further relates to compositions comprising nuclease resistant antibacterial oligonucleotides. These compositions generally comprise the oligonucleotide (or a mixture of oligonucleotides) and a physiologically acceptable carrier. After administration, the oligonucleotides enter the bacterial cell and bind to the target. The target may be a polynucleotide where hybridization to the oligonucleotide results in an inability of the polynucleotides to serve as templates for their encoded products. When the target is a protein, the bound oligonucleotide protein complex is inhibited relative to normal protein function (aptameric effect). As a result, growth of the bacteria are inhibited and the effects of the bacteria on the animal are less than they would have been if the oligonucleotides had not been administered.

Optionally, the presently described antibacterial oligonucleotides may be formulated with a variety of physiological carrier molecules. For example, the antibacterial oligonucleotides may be combined with a lipid (or cationic lipid), the resulting oligonucleotide/lipid emulsion, or liposomal suspension may, *inter alia*, effectively increase the *in vivo* half-life of the oligonucleotide. The use of cationic, anionic, and/or neutral lipid compositions or liposomes is generally described in International Publications Nos. WO 90/14074, WO 91/16024, WO 91/17424, Pat. No. 4,897,355, herein incorporated by reference.

The antibacterial oligonucleotides of the present invention may also be introduced into bacteria after being complexed with cationic lipids such as DOTMA (which may or may not form liposomes) which complex is then contacted with the target cells. Suitable cationic lipids include, but are not limited to, N-(2,3-di(9-(Z)-octadecenyloxyl))-prop-1-yl-N,N,N-trimethylammonium (DOTMA) and its salts, 1-O-oleyl-2-O-oleyl-3-dimethylaminopropyl-β-hydroxyethylammonium and its salts and 2,2-bis (oleyloxy)-3-(trimethylammonio) propane and its salts. By assembling the antibacterial oligonucleotides

into lipid-associated structures, the antibacterial oligonucleotides may be targeted to specific bacterial cell types by the incorporation of suitable targeting agents (*i.e.*, specific antibodies or receptors) into the oligonucleotide/lipid complex.

In another embodiment, the presently described purified oligonucleotides may be complexed with additional antibacterial agents. Additionally, the described nuclease resistant antibacterial oligonucleotides may also be linked to a conventional antibiotic or other chemical group that inhibits bacterial gene expression.

Having a demonstrated activity *in vitro*, the presently described antibacterial oligonucleotides are also contemplated to be effective in combating bacterial contamination of laboratory cultures, consumables (food or beverage preparations), or industrial processes.

4.1. Definitions

For the purposes of the present disclosure, the term "oligonucleotide" typically refers to a molecule comprising from about 8 to about 80 nucleotides, preferably about 15 to about 35 nucleotides, including polymers of ribonucleotides, deoxyribonucleotides, or both, with the ribonucleotide and/or deoxyribonucleotides being connected together via 5' to 3' linkages that may include any of the linkages known in the oligonucleotide art (including, for example, oligonucleotides comprising 5' to 2' linkages). In general, longer oligonucleotides (about 50 nucleotides) display enhanced targeting specificity but may be less efficient gaining entry to the target bacterium. Conversely, shorter oligonucleotides may more easily permeate the target bacteria, but may display a tendency to nonspecifically associate with host sequences and create a bystander effect or have no effect at all. Additionally, shorter oligonucleotides may less efficiently bind to, and thus nonspecifically inhibit, bacterial target sequences. For example, shorter antisense oligonucleotides (6mers to 7mers)

may prove less efficient at specifically binding the target mRNA, and may prove less efficient at activating RNase H activity. Shorter oligonucleotides may also effect host gene expression in a nonspecific, and thus undesirable, manner.

In spite of the above, the present application additionally contemplates relatively short oligonucleotide sequences (6mers to 7mers) having the desired antibacterial effects, and preferably broad-spectrum antibacterial effects, while exhibiting few adverse side effects in the host. In fact, an example of a short (6mer) oligonucleotide is provided below that exhibits significant antibacterial activity and is contemplated as a specific example of a preparation of an antibacterial oligonucleotide that functionally defines the lower size limit of the present invention. Given that the present invention specifically contemplates short oligonucleotides with demonstrated antibacterial function, the short oligonucleotides of the present invention specifically exclude short inoperative oligonucleotides such as AGGAGGT or GGAG.

Accordingly, additional embodiments of the present invention include relatively short (e.g. 6mers) oligonucleotides that have been identified by using the presently disclosed methods of synthesis in conjunction with standard antibacterial assays while gradually deleting bases from oligonucleotides with established antibacterial activity in order to define short antibacterial "core" sequences.

A particular embodiment of the present application contemplates oligonucleotides that have been modified to enhance the specificity of binding. Increased specificity allows for shorter oligonucleotides having the desirable features of both long and short oligonucleotides.

The presently described oligonucleotides may be constructed using either conventional bases (adenosine, cytosine, guanosine, thymidine, xanthine, inosine, or uridine) or any other modified bases, or base analogues that allow an oligonucleotide comprising such analogues to retain its ability to hybridize to a complementary nucleotide

sequence. Examples of such non-naturally occurring bases that are capable of forming base-pairing relationships with naturally occurring nucleotide bases include, but are not limited to, aza and deaza pyrimidine analogues, aza and deaza purine analogues as well as other heterocyclic base analogues, wherein one or more of the carbon and nitrogen atoms of the purine and pyrimidine rings have been substituted by heteroatoms, *e.g.*, oxygen, sulfur, selenium, phosphorus, and the like.

Modified oligonucleotides, nuclease resistant oligonucleotides, and antisense oligonucleotides are also meant to be encompassed by this definition. The term "oligonucleotide" is meant to encompass all of the foregoing, unless the context dictates otherwise.

The term "modified oligonucleotide" refers to oligonucleotides that include one or more modifications of the nucleic acid bases, sugar moieties, internucleoside phosphate linkages, as well as molecules having added substituents, such as diamines, cholesteryl or other lipophilic groups, or a combination of modifications at these sites. The internucleoside phosphate linkages can be phosphodiester, phosphotriester, phosphoramidate, siloxane, carbonate, carboxymethylester, acetamidate, carbamate, thioether, bridged phosphoramidate, bridged methylene phosphonate, bridged phosphoramidate, bridged phosphoramidate, bridged methylene phosphonate, phosphorothioate, methylphosphonate, phosphorodithioate, bridged phosphorothioate and/or sulfone internucleotide linkages, or 3'-3' or 5'-5' linkages, and combinations of such similar linkages (to produce mixed backbone modified oligonucleotides). The modifications can be internal or at the end(s) of the oligonucleotide molecule and can include additions to the molecule of the internucleoside phosphate linkages, such as cholesteryl, diamine compounds with varying numbers of carbon residues between amino groups and terminal ribose, deoxyribose and phosphate modifications which cleave or cross-link to the opposite chains or to associated enzymes

or other proteins. Electrophilic groups such as ribose-dialdehyde could covalently link with an epsilon amino group of the lysyl-residue of such a protein. A nucleophilic group such as n-ethylmaleimide tethered to an oligomer could covalently attach to the 5' end of an mRNA or to another electrophilic site. The term modified oligonucleotides also includes oligonucleotides comprising modifications to the sugar moieties such as 2'-substituted ribonucleotides, or deoxyribonucleotide monomers, any of which are connected together via 5' to 3' linkages. The term "modified oligonucleotide" is meant to encompass all of the foregoing, unless the context dictates otherwise, and also refers to oligonucleotides comprising chemical groups (*e.g.*, sugar molecules, amino acids, etc.) that may improve the antibacterial activity of the oligonucleotide.

The term "oligonucleotide backbone" refers to any and all means of chemically linking nucleotides such that oligonucleotides result that are capable of base-pairing or otherwise hybridizing, or interacting with a bacterial target sequence in a more-or-less sequence specific manner.

The term "purified oligonucleotide" refers to an oligonucleotide that has been isolated so as to be substantially free of, *inter alia*, incomplete oligonucleotide products produced during the synthesis of the desired oligonucleotide. Preferably, a purified oligonucleotide will also be substantially free of contaminants which may hinder or otherwise mask the antibacterial activity of the oligonucleotide. In general, where an oligonucleotide is able to bind to, or gain entry and inhibit the growth of a bacteria, it shall be deemed as substantially free of contaminants that hinder antibacterial activity. One example of a method to produce such purified oligonucleotides is described herein. In particular, an oligonucleotide preparation shall generally be considered substantially free of adverse contaminants (e.g., contaminants that hinder the measured antibacterial activity of the nucleotides such as alkyl amines, alkyl ammonium groups, or agents that block

oligonucleotide entry, etc.) when the sample proves effective in an *in vitro* MIC assay to an extent that is displays more than about twice, and preferably about five times, and most preferably at least about an order of magnitude greater antibacterial activity than a corresponding preparation that has not been treated to remove the adverse contaminants. Typically, an oligonucleotide preparation shall preferably be considered substantially free of adverse contaminants when the levels of contaminants in a sample are reduced to about 1/20th of the levels found in unpurified (or intermediately purified) samples, more typically about 1/50th of the levels found in unpurified samples, and preferably less than about 1/100th of the levels found in intermediately or unpurified samples of oligonucleotide.

Alternatively, an antibacterial oligonucleotide preparation may generally be considered free of adverse contaminants when the composition is about 95 percent free, and specifically about 99 percent free of contaminating alkyl amines, alkyl ammonium groups, or a mixture thereof as compared to unpurified crude or intermediately purified samples of the oligonucleotide preparation (as measured by conductivity, mass spectroscopy, or the extent to which a given oligonucleotide preparation retains antibacterial activity).

The term "substantially nuclease resistant" refers to oligonucleotides that are resistant to nuclease degradation, as compared to unmodified oligonucleotides, and include, but are not limited to oligonucleotides with modified backbones, such as, for example, phosphorothioates, methylphosphonates, ethylphosphotriesters, 2'-O-methylphosphorothioates, 2'-O-methyl-p-ethoxy ribonucleotide, 2'-O-methyl ribonucleosides, methyl carbamates, and methyl carbonates, inverted bases or chimeric versions of these backbones. Typically, the relative nuclease resistance of an oligonucleotide will be measured by comparing the percent digestion of a resistant oligonucleotide with the percent digestion of its unmodified counterpart (i.e., a corresponding oligonucleotide with

"normal" backbone, bases, and phosphodiester linkage). Such nuclease resistance tests generally add a given concentration of oligonucleotide (e.g., about 121 μmolar) to a given amount of nuclease S1 (at about 0.05 units per ml final concentration in the reaction), P1 (at about 0.05 units per ml final concentration in the reaction), SVP (at about 0.05 units per ml final concentration in the reaction), Micrococcal Nuclease (at about 0.5 units per ml final concentration in the reaction), etc., and measure the percent degradation (all reactions are incubated at about 37°C in the buffer appropriate for each nuclease. For example, S1 nuclease digestion conditions are typically 30 mM sodium acetate (pH 4.5), 50 mM NaCl, 1 mM $ZnCl_2$, 5% Glycerol; P1 nuclease digestion conditions are typically 30 mM sodium acetate (pH 5.3), 0.2 mM $ZnCl_2$; SVP digestion conditions were 100 mM Tris (pH 8.9) 100 mM NaCl, 14 mM $MgCl_2$; and Micrococcal nuclease digestion conditions are typically 50 mM sodium borate (pH 8.8), 5 mM NaCl, 2.5 mM $CaCl_2$). Percent degradation may be determined by using analytical HPLC to assess the loss of full length oligonucleotide, or by any other suitable methods (e.g., by visualizing the products on a sequencing gel using staining, autoradiography, fluorescence, etc., or measuring a shift in optical density). Degradation is generally measured as a function of time.

Generally, a substantially nuclease resistant oligonucleotide will be at least about 25% more resistant to nuclease degradation than an unmodified oligonucleotide with a corresponding sequence, typically at least about 50% more resistant, preferably about 75% more resistant, and more preferably at least about an order of magnitude more resistant after 15 minutes of nuclease exposure.

The term "targeted to a bacterial sequence" refers to the fact that the presently described antibacterial oligonucleotides are substantially homologous, otherwise complementary, or capable of associating with a target bacterial sequence. By associating with the target bacterial sequence, the presently described antibacterial

oligonucleotides are able to disrupt or inhibit the normal function of the target sequence, and hence inhibit bacterial cell division. In general, the antibacterial oligonucleotides will associate or bind to the target bacterial sequence and inhibit the function of the sequence by an antisense mechanism, an antigene (triplex) mechanism, or by stearic hindrance. Furthermore, the oligonucleotides can function through an aptameric mechanism by binding to nucleic acid binding proteins. For the purposes of the present invention, the term "aptamer" shall refer to oligonucleotides that are capable of binding or otherwise interacting with peptides, polypeptides, or proteins in a manner that effects the normal function of the peptide, polypeptide, or protein.

In order for the presently described antibacterial oligonucleotides to recycle their antibacterial activity, the oligonucleotides will generally associate with bacterial target sequences with an avidity sufficient to elicit an antibacterial effect, yet weak enough to allow the oligonucleotide to disassociate from the reaction products (*e.g.*, after messenger degradation, etc.) and subsequently target another molecule. One method of reducing the binding avidity, or relaxing the binding specificity, of an oligonucleotide is to truncate, or delete, a portion of the oligonucleotide.

Alternatively, another method of relaxing the binding avidity of an oligonucleotide comprises engineering a percentage of miss-match (or more-or-less neutral match, e.g., G-U base pairs) into the antibacterial nucleotide sequence. By reducing the net homology of a sequence, one effectively allows for antibacterial activity while increasing the kinetics of disassociation. Accordingly, an additional embodiment of the presently claimed methods and oligonucleotides are relaxed-specificity antibacterial oligonucleotides which comprise sequence miss-matches (with the corresponding target sequence) of up to about 60 percent, often about 35 percent, and preferably about 20 percent, or

less. In spite of the percentage miss-match, the relaxed-specificity oligonucleotides remain capable of associating with bacterial target sequences under physiological temperatures and conditions. For the purposes of the present invention, the term "miss-match" shall apply to all Watson and Crick polynucleotide base-pairs, other than A:T, G:C, and A:U, and the inverses thereof.

Furthermore, one of ordinary skill will appreciate that the maximally tolerated percentage miss-match may vary depending on the G/C content of the oligonucleotide. In general, an A/T-rich sequence may tolerate a fairly high percentage of miss-match where the G/C base pairs have been retained. In any event, the amount of sequence miss-match should not be such that undue side effects result in the host.

Additionally, given the reduced charge associated with oligonucleotides comprising partially or fully substituted chemical backbones, it is to be understood that such oligonucleotides may retain the ability to bind target bacterial sequence under physiological conditions although comprising a greater amount of sequence miss-match than may be tolerated by conventional oligonucleotides.

An additional embodiment of the present invention is antibacterial oligonucleotides that are capable of inhibiting bacterial growth by cross reacting with a variety of both known and unknown bacterial target sequences. For the purposes of the present disclosure, the term "cross reactive antibacterial oligonucleotide" shall refer to an oligonucleotide sequence that inhibits bacterial growth by interacting with bacterial sequences that may share less than 100 percent sequence homology, and preferably at least about 50 percent sequence homology, with the oligonucleotide. Examples of such a cross reactive antibacterial activity include: instances where heterologous, similar, and homologous bacterial sequences are bound and affected by an oligonucleotide that is targeted to a related sequence; instances where an antibacterial oligonucleotide is able to

interact with bacterial sequences that share a sufficient percentage of otherwise random sequence complementarity (*e.g.*, short, interspersed regions of high sequence complementarity, etc.) with the oligonucleotide such that bacterial growth is inhibited; and instances where a given antibacterial oligonucleotide is able to inhibit bacterial growth although all or some of the affected bacterial target sequences are unknown (this includes instances where the cross reactive oligonucleotide has up to 100% homology with an unknown target DNA sequence). Target sequences comprised within conserved or related control regions, which are often noncoding, are deemed to constitute particularly effective targets for cross reactive antibacterial oligonucleotides that operate via an antigene mechanism.

A "functional equivalent" of the sequences disclosed in the Sequence Listing shall include any oligonucleotides comprising sequence that is at least about 25 percent sequence homologous, preferably about 33 percent sequence homologous, and more preferably at least about 50 percent homologous to any one of SEQ ID NOS. 1-176, and demonstrates at least about 30 percent, and preferably at least about 50 percent of the antibacterial activity of the corresponding oligonucleotide in the Sequence Listing when measured in an MIC assay.

The term "bacterial sequence" includes any and all forms of DNA, RNA or amino acid polymers (or oligomers) that are present in the cell.

The term "competent cells" refers to bacterial cells that have been manipulated in culture or otherwise chemically, osmotically, or thermally modified such that the cells bear an enhanced ability to internalize exogenous nucleic acid.

The term "pathogenic bacteria" refers to any and all bacteria that are, or have been, associated with clinical symptoms of disease in animals, including humans. The term "wild-type" bacteria refers to a bacteria that has not been modified either chemically or genetically in any way

whatsoever (other than growth in culture medium). In particular, a "wild-type" bacteria shall not be genetically modified such that the bacteria has an enhanced permeability to macromolecules or biological polymers or oligomers.

The term "antisense oligonucleotide" refers to an oligonucleotide that has a sequence that is substantially complementary to a target DNA or mRNA, so that the antisense oligonucleotide will hybridize in a complementary fashion to the DNA or mRNA to form a complex by Watson-Crick base pairing. Generally, the antisense oligonucleotide will bind the complementary target sequence with an avidity, *in vivo*, sufficient to inhibit the normal function of target sequence.

The term "bacteriostatic oligonucleotide" refers to oligonucleotides that inhibit or retard the growth of bacteria either *in vitro* or *in vivo*.

The term "bactericidal oligonucleotide" refers to oligonucleotides that directly, or indirectly, cause the death of bacteria either *in vitro* or *in vivo*.

The term "Gram negative bacteria" refers to the inability of bacteria to resist decolorization with alcohol after being treated with Gram's crystal violet stain. However, following decolorization, these bacteria can be readily counter-stained with safranin, imparting a pink or red color to the bacterium when viewed by light microscopy. This reaction is usually an indication that the bacterium's outer structure consists of a cytoplasmic membrane (inner), which is surrounded by a relatively thin peptidoglycan layer, which in turn, is surrounded by an outer membrane. Typical examples of Gram negative bacteria include, but are not limited to, *Escherichia, Salmonella, Edwardsiella, Arizona, Citrobacter, Enterobacter, Proteus, Yersinia, Klyvera, Klebsiella, Neiserria, Vibrio, Pasturella, Haemophilus, Pseudomonas, Moraxella, Eikenella, Fusobacterium, Acidominococcus, Actinobacillus, Cardiobacterium, Serratia, Providencia, Erwinia, Tatumella, Shigella, Branhamella, Aeromonas, Francisella, Gardnerella, Alcalígenes, Kingella, Agrobacterium, Leptotrichia, Megasphaera, Capnocytophaga,*

Cromobacterium, Hafnia, Morganella, Pectobacterium, Cadecea, Helicobacter, Morococcus, Pleisiomonas, Bordetella, Brucella, Achromobacter, Flavobacterium, Bacteroides, Veillonella, Streptobacillus, Pneumococcus, and *Calymmatobacterium.*

The term "Gram positive bacteria" refers to the ability of bacteria to resist decolorization with alcohol after treatment with Gram's crystal violet stain, imparting a violet color to the bacterium when viewed by light microscopy. This reaction is usually an indication that the bacterium's outer structure consists of a cytoplasmic membrane surrounded by a thick, rigid bacterial cell wall mainly comprised of peptidoglycan (murein). Typical examples of Gram positive bacteria include, but are not limited to, *Aerococcus, Listeria, Streptomyces, Actinomadura, Lactobacillus, Eubacterium, Arachnia, Mycobacterium, Peptostreptococcus, Staphylococcus, Corynebacterium, Erysipelothrix, Dermatophilus, Rhodococcus, Bifodobacterium, Lactobacillus, Streptococcus, Bacillus, Peptococcus, Micrococcus, Kurthia, Nocardia, Nocardiopsis, Rothia, Propionibacterium, Actinomyces, Enterococcus,* and *Clostridia.*

Additionally, the presently described antibacterial oligonucleotides may be effective against bacteria including, but not limited to, *Campylobacter, Spirillium, Borrelia, Treponema, Leptospira, Legionella,* and *Chlamydia.*

The term "mycobacterium" refers to any and all strains of bacteria drawn from the group comprising: *Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium leprae, Mycobacterium avium-intracellulare, Mycobacterium kansasii, Mycobacterium scrofulsceum, Mycobacterium marinum, Mycobacterium fortuitum, Mycobacterium ulcerans, Mycobacterium chelonae, Mycobacterium paratuberculosis, Mycobacterium xenopi, Mycobacterium simiae,* or other mycobacteria falling within the Runyon groups I-IV as described in Runyon, Med. Clin. North Amer. 43:273-290 (1959), or Mandell *et al.*, 1990, Principles and Practice of Infectious Disease 3rd. ed., Churchill Livingstone Inc., New York, N.Y. 10036, herein incorporated by reference.

The term "MIC test" refers to a National Committee on Clinical Laboratory Standards ("NCCLS") approved test for determining the minimum inhibitory concentrations ("MIC") of bacteria by broth dilution. This term includes the use of this test for determining the percent inhibition of bacterial growth by the oligonucleotides of the invention.

The term "transport" refers to the movement of the oligonucleotides of the invention from outside the bacterial cell across the bacterial cell's outer-structure and into the bacterial cell's cytoplasm.

The term "virulence factor" refers to bacterial products which contribute to the pathogenicity of a bacteria, such as, for example, antibiotic resistance factors, toxins (exo- and endo-), adherence factors that recognize host tissues, extracellular receptors, bacterial iron-binding proteins, and surface modifications that allow the bacteria to escape the immune system (*e.g.*, polysaccharide coats or capsules).

The term "labeled oligonucleotides" refers to oligonucleotides that have been modified to allow a determination of the presence or amount of the oligonucleotide. Typical labels include, for example, radioisotopes, biotin, and enzymes (such as luciferase, or β-galactosidase).

The term "stringent conditions" generally refers to hybridization conditions that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% SDS at 50°C.; (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M Sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 g/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS. The above examples of hybridization conditions are merely provided for purposes of exemplification and not

limitation. One of ordinary skill will appreciate that stringency may generally be reduced by increasing the salt content present during hybridization and washing, reducing the temperature, or a combination thereof. A more thorough treatise of such routine molecular biology techniques may be found in Sambrook *et al.*, Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, Vols. 1-3 (1989), and periodic updates thereof, herein incorporated by reference.

4.2. Synthesis Of Oligonucleotides

The described oligonucleotides may be partially or fully substituted with any of a broad variety of chemical groups or linkages including, but not limited to: phosphoramidates, phosphorothioates; p-ethoxy; alkyl phosphonate; 2'-O-methyl; 2' modified RNA; morpholino groups; phosphate esters; dithioates; 5' thio groups; propyne groups; or chimerics of any combination of the above groups or linkages (or analogues thereof), or any other chemical modifications that leave the oligonucleotide capable of specifically binding to nucleic acid or protein.

Oligonucleotides, methylphosphonates, and phosphorothioates may be synthesized, using standard reagents and protocols, on an automated synthesizer utilizing methods that are well known in the art, such as, for example, those disclosed in Stec *et al.*, J. Am. Chem. Soc. 106:6077-6089 (1984), Stec *et al.*, J. Org. Chem. 50(20):3908-3913 (1985), Stec *et al.*, J. Chromatog. 326:263-280 (1985), LaPlanche *et al.*, Nuc. Acid. Res. 14(22):9081-9093 (1986), and Fasman, G.D. Practical Handbook of Biochemistry and Molecular Biology, 1989, CRC Press, Boca Raton, Florida, herein incorporated by reference.

The principal criteria for designing nuclease resistant oligonucleotides are: (1) retention of sequence-specific base-pairing and triplex-forming interactions (*i.e.*, the ability to associate with bacterial target sequence such that bacterial growth is inhibited); (2) increasing nuclease

stability; (3) ease of synthesis and purification. The most common strategies to date have involved neutralizing the charge on the phosphodiester backbone by substitution at, or replacement of, the phosphodiester moiety, conjugating moieties at the 3' and/or 5' terminus, and substitutions at the 2'-position of ribose and deoxyribose. In particular, the addition of a 3'-3' or 5'-5' internucleotidic linkages at either end of the oligonucleotide, may inhibit degradation by the respective exonuclease (Seliger *et al.*, 1991, Nucleosides and Nucleotides, 10:463-477). Additionally, several new strategies have recently emerged that utilize peptide interlinkages.

The synthesis of phosphoramidates is disclosed in Agrawal *et al.*, Proc. Natl. Acad. Sci. USA 85:7079-7083 (1988). The preparation of phosphoramidates modified with several methoxyethyl phosphoramidate internucleoside linkages is disclosed in Dagle *et al.*, Nucl. Acids Res. 18(6):4751-4757 (1990). These modified oligonucleotides are highly resistant to nucleolytic degradation and can also serve as a substrate for RNase H (which degrades the RNA component of a DNA/RNA hybrid).

An approach for synthesizing formacetal linked dinucleosides is disclosed by Quaedflieg *et al.*, Tetrahedron Lett. 33(21):3081-3084 (1992).

The synthesis and binding properties of pyrimidine oligonucleotides containing alternating modified and natural internucleoside linkages, formacetal and thioformacetal, is disclosed by Jones *et al.*, J. Org. Chem. 58:2983-2991 (1993). The thioformacetal modified oligodeoxynucleotides (ODN) displayed high affinity and specificity for both single-stranded RNA and double-stranded DNA targets, indicating that this linkage is promising for both antisense and triplex (antigene) therapeutic applications.

The synthesis of hexanucleotide analogues containing internucleotide diisopropylsilyl linkages is disclosed by Cormier and Ogilvie, Nucl. Acids Res. 16(10):4583-4594 (1988). These oligonucleotides were not readily soluble in

water. It has been suggested that inserting terminal or internal phosphodiester groups, or highly hydrophilic groups would increase water solubility of these compounds.

The synthesis of acetamidate linked oligomers of mean chain length 10-13 is disclosed by Gait *et al.*, J. Chem. Soc., Perkin Trans. 1:1684 (1974).

The synthesis of dinucleotides and trinucleotides modified with carbamate (-OCO-NH-) bonds is disclosed by Mungall and Kaiser, J. Org. Chem. 42(4):703-706 (1977). The carbamate linkage was found to be stable toward acid and base hydrolysis, as well as toward nucleases.

The synthesis of oligonucleotides with dimethylene-sulfide ($-CH_2-S-CH_2$), -sulfoxide ($-CH_2-SO-CH_2$), and -sulfone ($-CH_2-SO_2-CH_2$) groups replacing phosphodiester linkages is reported by Schneider and Brenner, Tetrahedron Lett. 31(3):335-338 (1990); Huang *et al.*, J. Org. Chem. 56:3869-3882 (1991); Musicki *et al.*, Tetrahedron Lett. 32(10):1267-1270 (1991); Huang *et al.*, Tetrahedron Lett. 33(19):2657-2660 (1992); and Reynolds *et al.*, J. Org. Chem. 57:2983-2985 (1992).

The synthesis of 2'-O-alkyloligoribonucleotides, where the alkyl groups are methyl, butyl, allyl or 3,3-dimethylallyl is reviewed by Lamond, Biochem. Soc. Trans. 21:1-8 (1993). Oligomers comprised of the modified linkages formed stable duplexes that exhibited a higher Tm (upon binding complementary RNA) than unmodified RNA-RNA duplexes. Oligonucleotides containing the modified linkages are nuclease resistant. It was found that binding of allyl-modified oligomers to A/U rich mRNA sequences (typical of snRNAs) could be improved by incorporating the modified base 2-aminoadenine in the modified probe.

The synthesis of 2'-deoxyuridine analogues carrying an amino linker at the 1'-position of deoxyribose is disclosed by Ono *et al.*, Bioconjugate Chem. 4:499-508 (1993). The uridine analogues were incorporated into oligonucleotides and intercalating groups such as anthraquinone and pyrene derivatives that were attached to the amino group of the

linker. Several oligonucleotides were synthesized that incorporated the analogues at several different sequence positions. Duplexes formed with the analogues were more stable than unmodified duplexes. Also, the oligonucleotide analogues were resistant to exo- and endonuclease degradation. Moreover, duplexes formed with the analogues were capable of activating RNase H. The authors suggested that the bulky group attached at the C1'-position stearically masked the phosphodiester linkage from nuclease attack.

The synthesis of uniformly modified 2'-deoxy-2'-fluoro phosphorothioate oligonucleotides is disclosed by Kawasai *et al.*, J. Med. Chem. 36:831-841 (1993). Since 2'-deoxy-2-fluororibose adopts the 3'-endo conformation, it was hypothesized that deoxy oligomers modified at the 2'-position with fluorine would adopt more uniform and more stable duplexes with RNA. The modified oligomers were found to possess thermal stabilities similar to or higher than those of the corresponding RNA duplexes. The modified oligomers demonstrated resistance to nucleases, but did not activate RNase H.

A description of the synthesis of p-ethoxy-linked oligonucleotides may be found, *inter alia*, in application Ser. No. 08/065,016, filed May 24, 1993, herein incorporated by reference. The synthesis of inverted bases is described in Seliger *et al.*.

Additional antibacterial oligomers may be adapted from the polynucleotide binding polymers and backbones described in Pat. Nos. 5,034,506, 5,142,047, 5,166,315, 5,185,444, 5,470,974, and 5,235,033, which are herein incorporated by reference.

The synthesis of oligonucleotides containing any of the above internucleotide linkages is well known to those skilled in the art, as is further illustrated in articles by Uhlmann *et al.*, Chem. Rev. 90:543-584 (1990), and Schneider *et al.*, Tetrahedron Lett. 31:335 (1990). See also Reissue Pat. No. 34,069, herein incorporated by reference.

4.2.1 Oligonucleotides Comprising Modified Nucleosides

α-Anomeric Nucleoside Units. The synthesis of a octathymidylate comprised of α-anomers is disclosed by Thuong *et al.*, Proc. Natl. Acad. Sci. USA 84:5129-5133 (1987). The modified oligomer binds to complementary sequences containing naturally occurring β anomers. A 3'-acridine linked α-anomer was also prepared. This analogue also demonstrated sequence-specific binding. The α-anomers demonstrated nuclease stability, independently of whether linked to acridine or not.

Base-Modified Nucleoside Units. The synthesis of a base analogue designed to recognize T-A and G-C Watson-Crick base pairs to facilitate sequence-specific triplex formation is disclosed by Griffin *et al.*, J. Am. Chem. Soc. 114:7976-7982 (1992).

4.3. Purification Of Oligonucleotides

The present disclosure teaches that the relative purity of an antibacterial oligonucleotide may profoundly impact its antibacterial activity. As discussed in greater detail below, the antibacterial activity of an oligonucleotide may be enhanced by at least 60 percent after it has been subject to an appropriate purification protocol. It is particularly important that purification remove contaminants that either obstruct the uptake of the oligonucleotides or mask the antibacterial activity of the oligonucleotides by, for example, stimulating bacterial growth.

A variety of standard methods were used to purify/produce the presently described antibacterial oligonucleotides. In brief, the antibacterial oligonucleotides of the present invention were purified by chromatography on commercially available reverse phase (for example, see the RAININ Instrument Co., Inc. instruction manual for the DYNAMAX®-300A, PureDNA™ reverse-phase columns, 1989, or current updates thereof, herein incorporated by reference) or ion exchange media (see generally, Warren and Vella, 1994, "Analysis and Purification of Synthetic

Oligonucleotides by High-Performance Liquid Chromatography", *In* Methods in Molecular Biology, vol. 26: Protocols for Oligonucleotide Conjugates, S. Agrawal ed., Humana Press, Inc., Totowa, NJ; Aharon *et al.*, 1993, J. Chrom. 698:293-301; and Millipore Technical Bulletin, 1992, "Antisense DNA: Synthesis, Purification, and Analysis"). Peak fractions were combined and the samples were desalted and concentrated by alcohol (ethanol, butanol, isopropanol, and isomers and mixtures thereof, etc.) precipitation, diafiltration, or gel filtration followed by lyophilization, or solvent evaporation under vacuum in commercially available instrumentation such as, for example, a Savant Speed Vac.

Oligonucleotides of the invention were dissolved in pyrogen free, sterile, physiological saline (*i.e.*, 0.85% saline) and sterile filtered through 0.2 micron pyrogen free filters.

4.4. Oligonucleotides As Antibiotics

The principal criteria for designing antisense oligonucleotides for treating bacterial infections are: (1) retention of sequence-specific base-pairing and triplex-forming interactions; (2) increasing nuclease stability; (3) increasing the extent or kinetics of entry into the target cell; (4) activating RNase H (while a consideration, a given oligonucleotide's ability to activate RNase H is not strictly required to observe antibacterial activity); and (5) ease of synthesis and purification.

Although exquisite sequence specificity may be preferred in some instances, the presently described oligonucleotides are capable of specifically inhibiting bacterial growth as long as they remain capable of associating with the target sequence under the relevant conditions. For example, the use of oligonucleotides to degrade RNA simply requires that the oligonucleotide associate (with at least a four base match) with the bacterial RNA long enough to activate RNase H. Thus, oligonucleotides that harbor relaxed sequence specificity are deemed sufficient to activate RNase H. In

fact, because not all bacterial target sequences are known, applications are contemplated where the antibacterial oligonucleotide provides the desired inhibitory effect although not specifically targeted, or homologous, to a given bacterial gene.

Modified oligonucleotides that activate RNAse H are advantageous because such oligonucleotides will hybridize to their target mRNAs and create a substrate that can be digested by RNase H. RNase H digestion destroys the target mRNA, and thus, these oligonucleotides prevent the translation of the target mRNA. Accordingly, protein expression is inhibited either by the enzymatic destruction of the target mRNA, or by the oligonucleotide physically blocking translation (*i.e.*, after the oligonucleotide directly associates with ribosomal sequence).

Although RNase H activation is a factor in the design of antibacterial oligonucleotides, many antibacterial oligonucleotides (e.g., ribonucleotides targeting bacterial RNA) are not designed to activate RNase H. Typically, modified oligonucleotides that are connected by stretches of unmodified phosphodiester linkages comprising at least about four nucleotides to about seven nucleotides should retain the ability to activate RNase H. Also, it has been observed that phosphorothioate ribonucleotides can also activate RNAase H digestion. The differential specificity of mammalian RNase H (minimum of 5 bases) and bacterial RNAase (4 bases) affords a means of selectively targeting bacterial genes that may have strong sequence homology with certain animal genes.

Also contemplated are modified oligonucleotides that can form triplexes with duplex DNA (antigene oligonucleotides), and oligonucleotides that can be used as ribozymes.

Another embodiment of the presently described antibacterial oligonucleotides is aptameric oligomers that are capable of effectively mimicking protein domains and exerting an antibacterial effect by directly associating with bacterial proteins or structures.

Additionally, antibacterial oligonucleotides may exert a therapeutic effect by specifically binding and deactivating cellular machinery. For example, the presently described oligonucleotides may directly bind ribosomal sequences and inhibit translation by stearically hindering translation initiation, elongation, disassociation, or by directly destabilizing the structure of the bacterial ribosomes.

Antibiotic resistance is often caused by the presence of resistance factors that render an antibiotic ineffective. By targeting resistance factors, the presently described oligonucleotides may render an otherwise antibiotic resistant organism sensitive to conventional antibiotics. Accordingly, another embodiment of the present invention is the use of antibacterial oligonucleotides in conjunction with conventional antibiotics.

Another embodiment of the present invention involves the use of the presently described oligonucleotides to inhibit the expression of genes whose products regulate the replication or transfer of bacterial genes. Additionally, given that antibiotic resistance genes or other virulence factors are often encoded by plasmids, antibacterial oligonucleotides targeted against plasmid replication, transfer (by conjugative transfer), or gene expression are particularly of interest. Similarly, antibacterial oligonucleotides are contemplated that are capable of inhibiting the expression and transfer of genes encoded by transposable genetic elements (*e.g.*, transposons).

4.4.1. Selection Of Targets For Oligonucleotides: Gene/Operon Target Identification

Antisense oligonucleotides which target essential structural genes, metabolic pathway genes, or transport system genes will inhibit the growth of bacterial cells. For pathogenic bacteria, virulence factors such as, for example, genes encoding antibiotic resistance, toxins, adherence and invasion factors, pili or fimbriae, flagella, antigenic variation factors, and iron binding factors, are also

preferred targets. These targets should be pathogen specific, and thus oligonucleotides directed against these targets will preferably not harm either host cells, or the normal bacterial flora of the gut.

While some bacterial genes are expressed as individual transcripts, many are transcribed as part of a multicistronic unit or operon. Examples include the ribosomal protein operons, such as the *str* operon and the alpha operon in *Escherichia coli*. Where possible operon transcripts are targeted. Disruption of expression of a gene in the operon may also adversely effect the expression of other genes encoded within the same operon (often in operon transcripts the translation of the 5'-most genes are required for efficient translation of the downstream genes). In theory this could result in pleiotropic growth effects from a single oligonucleotide sequence. Specific genes and transcripts (whether expressed as part of an operon or independently) are targeted on the basis of their function in the cell. For example, the gene for glucose-6-phosphate dehydrogenase is central to sugar metabolism. Other genes may not be relevant in our normal assay system; disruption of lactose metabolism is expected to have only a minor effect, if any, on *Escherichia coli* growth in media containing a more readily available carbon source such as glucose.

Once a target gene or operon has been selected, a target region within the gene or operon sequence must be selected, for example, the start codon. An analysis of the sequences around the target sequence (*e.g.*, 5' untranslated region, start codon, internal sequence feature, termination codon, 3' untranslated region) is performed. This analysis generally encompasses a total of about 120 bases that flank the target sequence. This analysis further predicts the secondary structure of the antisense oligonucleotide, and can be performed using commercially available computer software. The extended target sequence is checked for regions of stable secondary structure. The positions of the bases predicted to be involved in the stem-and-loop structures should be marked

and the predicted Tm of the structures noted. Preferably, stem sequences should be avoided where possible. Moreover, predicted secondary structures with predicted melting temperature of 45°C or less are disregarded in this analysis.

A maximum oligonucleotide length is also selected, and the program identifies the clear regions (no stems, or the structures with the lowest melting temperatures), and also checks the loop melting temperatures for the generated oligonucleotides. Such programs are well known in the art and include, for example, the program *OligoTech* version 1.0 (Copyright© 1995, Oligos Etc. Inc. & Oligo Therapeutics Inc.).

The length of the flanking sequence to be analyzed may be increased if an oligonucleotide with a length of greater than 30 bases is selected. The transcription start site and termination site (or any attenuation sequence) are generally the most distal sequences that will be analyzed. On occasion, this may result in an analysis of about 190 or more bases of flanking sequence.

Potential oligonucleotide sequences that have high loop melting temperatures may be eliminated by the above analysis. Note that the melting temperatures for the loops obtained for the commercial programs may need to be adjusted for modified oligonucleotides since these oligonucleotides may have altered base pairing avidities.

Several additional characteristics of the oligonucleotides are also considered. Stable secondary structure (potentially stable under physiologic conditions), runs of a single base (*e.g.*, 4 or more A's), and sequences that potentially form stable homodimers are also eliminated if possible. (In cases where double-strand oligonucleotide is the desired end result, homodimers may be preferred.) The base composition of the oligonucleotide is also checked.

The two or three oligonucleotide sequences that most nearly meet the above criteria are selected. Using these final oligonucleotides, the program analyzes each sequence and notes loop melting temperatures for both the sense and

the antisense strands of the candidate sequences. This decreases the possibility of the computer analysis missing a potential problem structure.

The candidate sequences, selected as above, are searched for sequence matches in available sequence databases (for example, Genbank) using commercially available search software. The first search is against the bacterial sequence database(s). This allows the identification of other targets that may also be affected by the candidate sequence, and may also indicate which sequences are potentially effective across bacterial genera. Since many different bacterial genera have highly related genetic organizations or related gene sequences, a potential oligonucleotide may be effective against multiple bacterial genera. For example, the sequences of the *gyrA* genes of *Escherichia coli* and *Salmonella typhimurium* are essentially identical near the start codon.

Additionally, since bacterial translation occurs simultaneously with transcription, it may be generally preferable to target antisense oligonucleotides to bacterial sequences at or near the Shine-Delgarno site (ribosome binding site) or to the translation start site of the targeted transcript.

The second search is versus a database including human/primate sequences. Since these databases are still quite limited (relative to the entire amount of sequence data in the genome), databases generally including mammalian sequences should be searched. Oligonucleotides that have high specificity matches to relevant mammalian sequences should be eliminated from initial consideration. (Note: that they may be re-included after further evaluation of the possible target sequences.)

As a consequence of the incomplete nature of the data bases comprising bacterial, primate, rodent, and mammalian sequences, this method cannot ensure that all potential targets or conflicts are identified. However, as sequence data accumulates, this method will allow an experienced

practitioner of the art to identify targets and select oligonucleotide sequences for use in the methods of the invention.

4.5. Bacterial Inhibition Assay: MIC Test

Despite some limitations of *in vitro* susceptibility tests, the clinical data indicate that there is good correlation between MIC test results and *in vivo* efficacy of antibiotics. Murray, P., Antimicrobial Susceptibility Testing, (Poupard *et al.*, eds.), Plenum Press, NY, 1994; Knudsen *et al.*, Antimicrob. Agents Chemother. 39(6):1253-1258 (1995).

Accordingly, the presently described antibacterial oligonucleotides were tested for antibacterial activity *in vitro*. Prior to use *in vivo*, a given antibacterial oligonucleotide will have demonstrated antibacterial activity *in vitro* against a pathogenic bacteria. Generally, the *in vitro* antibacterial activity of an oligonucleotide will be tested using a standard bacterial inhibition assay, or MIC test (see National Committee on Clinical Laboratory Standards "Performance Standards for Antimicrobial Susceptibility Testing" NCCLS Document M100-S5 Vol. 14, No. 16, December 1994, herein incorporated by reference).

4.5.1. Variations On The Standard MIC Test

Cells that are growing exponentially *in vitro* are generally not representative of cells in clinical infections where nutrients may be limited and the cells are dividing slowly or not at all, *i.e.*, the cells are in stationary phase. Starved stationary phase cells undergo a series of morphological and physiological changes that distinguish them from cells in exponential growth. These changes ensure the prolonged survival of the cells by reducing endogenous metabolism and preparing the cells for possibly adverse conditions.

Further, there is a specific interrelation between the growth rate of bacterial cells and the sensitivity of the

cells to chemicals, antibiotics, and host defenses. Thus, antibiotics developed and tested against laboratory cultures are often ineffective when directed against relatively slowly growing, clinical infections.

In an effort to address the issue of bacteria growing under starved conditions in a clinical setting, both fresh cultures and starved cultures of bacteria were used as inocula in standard MIC tests. Oligonucleotides with antibacterial activity proved effective regardless of the type of inoculum used in the MIC test.

The MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in the tubes or microdilution wells as detected by the unaided eye. Viewing devices intended to facilitate reading microdilution tests and recording of results may be used as long as there is no compromise in the ability to discern growth in the wells. The amount of growth in the wells or tubes containing the antibiotic should be compared with the amount of growth in the growth-control wells or tubes (no antibiotic) used in each set of tests when determining the growth and points.

The percent inhibition of an oligonucleotide as reported herein was the absorbance at 625 nanometers of a bacterial culture that was treated with the oligonucleotide divided by the absorbance at 625 nanometers (*i.e.*, O.D. 625) of a duplicate cell culture minus oligonucleotide (control); the resulting number was subtracted from 1, and multiplied by 100%. Small variations in the optical density readings at the lower detection limit of the assay may result in calculated inhibitions of greater than 100 percent. It is assumed that these calculations essentially represent 100 percent inhibition.

The concentration of target bacteria used in an MIC assay typically far exceeds the systemic concentrations of pathogenic bacteria that, with the possible exception of abscesses, are expected to be found *in vivo*. While even the presence of a single bacterium in bodily fluids is considered an indication of infection (John J. Sherris, Editor, Medical

Microbiology, An Introduction to Infectious Diseases, 2nd Edition, Elsevier, New York 1990), the precise number of bacteria/ml is not well quantified in human clinical infections (Kjeldfberg and Knight (3rd Edition), Body Fluids, ASCP Press, 1993). It is difficult to quantitate bacteria in body fluids as bacteria are constantly cleared by the immune system (Myrvik, Fundamentals of Medical Bacteriology, 1974, Lea & Febiger, Publishers). In addition, bacteria grow more slowly *in vivo* than *in vitro*, so this slow growth combined with the clearance by the immune system makes quantifying the number of bacteria difficult. In order to quantitate clearance of *Pneumococci* in the blood, Wilson (G.S. Wilson and A.A. Miles, Editors, Topley and Wilson's Principles of Bacteriology and Immunology, Williams & Wilkins, Publishers, 1964) reported a study where bacteria were intravenously injected into rabbits. It is evident from these data that if the immune system is unable to clear the bacteria from the blood, once the concentration of bacteria reaches 1.5×10^6 cfu per ml the animal will die. In light of the above discussion, the oligonucleotides need only arrest the growth of the bacteria until the immune system is capable of clearance. Furthermore, in an actual clinical situation, the concentration of bacteria/ml would be far lower than 1.5×10^6/ml, which represents a fatal concentration in Wilson's animal model.

In the presently described studies, the bacteria were grown over the period of the assays to an O.D. 600 of 0.1 as defined by the NCCLS. This represents approximately 1×10^8 concentration of bacteria which represents more bacteria/ml than would be required to cause death in a clinical setting.

4.5.2. Fastidious Organisms

The standard media used in the MIC tests described above for the rapidly growing aerobic pathogens (Mueller-Hinton medium) is not adequate for susceptibility testing of fastidious organisms. Where MIC tests are to be done using fastidious organisms, the medium, quality control procedures,

and interpretive criteria must be modified to fit each organism. For example, dilution tests for *Haemophilus influenzae* (using *Haemophilus* test medium), *Nisseria gonorrhoeae* (using GC agar base medium), and *Streptococcus pneumoniae* (using lysed horse blood-supplemented, cation-adjusted Mueller-Hinton broth) have been shown to be reliable methods. It is important to note that the direct inoculum suspension method of preparing the test inoculum must be used with these three species. The media and important technical aspects of testing several fastidious species are described in relevant sections above and outlined in NCCLS Doc. M7-A3, Vol. 13, No. 25, entitled "Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically - Third Edition; Approved Standard". Interpretive criteria for testing these three fastidious species can also be found in NCCLS Doc. M7-A3, Vol. 13, No. 25.

4.6. Antibacterial Activity *In vivo*

After demonstrating antibacterial activity *in vitro*, the antibacterial oligonucleotide will be tested for activity *in vivo*. In brief, an antibacterial oligonucleotide sequence (e.g., a phosphorothioate ODN) will be tested for antibiotic activity in a mammalian test subject, and preferably a murine test subject. Phosphorothioate ODNs have previously been tested in mammals (mice, rats, rhesus monkeys), and, when properly administered, have not been found to be significantly toxic. Prior to introduction *in vivo*, ODNs will be solubilized in sterile saline and serially-diluted to the desired test concentrations in sterile saline.

Bacteria. Bacterial pathogens to be used *in vivo* include, but are not limited to, *inter alia*, the drug-resistant *Escherichia coli* ATCC accession No. 25922, and *Staph. aureus* ATCC accession No. 13301. Generally, the target/test bacteria are cultured *in vitro* in Mueller-Hinton broth (BBL Microbiology Systems, Cockeysville, MD) for 18 hours at 37°C.

Typically, cultures of a test pathogen will be prepared by suspending colonies grown on solid medium (for example, trypticase soy agar plates) into 70 ml of Mueller-Hinton broth so that a culture with an optical density of about 0.1 at 540 nm results. Appropriate dilutions of the bacterial cells are then prepared in DPBS.

Animals. Typically, any acceptable animal model may be used to assess the efficacy of the antibacterial oligonucleotides. Additionally, experimental protocols and conditions will necessarily be adjusted as applicable depending on the bacterial pathogen being tested and the mode of infection. Accordingly, the following example is provided merely for purposes of exemplification and should not be deemed as limiting the present invention in any way whatsoever.

Six- to eight-week-old CD1 mice or NMRI mice, 24-28 g in size, are typically used in these studies. The CD1 strain of mouse has been used in the past for certain studies of infectious diseases and therapeutics (*e.g.*, Brogden *et al.*, (1986); Cavalieri *et al.*, (1991); Lister and Sanders, Antimicrob. Agents Chemother. 39:930-936 (1995)), as has the NMRI strain (Hof *et al.*, Infection 114:190-194 (1986)). Thus, both of the above strains are exemplary of well established infectious disease models that are also readily available to those of ordinary skill.

Typical animal tests comprise a minimum of about 5-8 animals in each treatment group (1 cage of 5 mice each) in order to demonstrate adequately the statistical reproducibility of a given experimental observation. By using at least about 5 test animals, one can compensate for variabilities such as differing growth rates of microorganisms in a given animal and any variables introduced by the repeated handling and injection of the animals.

Injection of microorganisms. Test animals are typically injected subcutaneously (SC) on the back (intrascapular) with approximately 0.3 ml of bacterial cell suspension in 1.5% liquified sterile tryptose phosphate agar held at 39°C

essentially as described by Hof et al. (1986) or I.P. with 5% mucin (Lister & Sanders, 1995).

Administration of Oligonucleotides. At the time of injection of bacteria or at various times after injection with the indicated microorganism, the test animals are treated by administration of a bolus injection of oligonucleotides at, for example, 0, 1.0, 2.5, 5.0 or 10.0 mg/kg (5 separate groups, one dose per group of 12 animals) to determine optimum therapeutic dose of a given antibacterial oligonucleotide. The oligonucleotide is generally administered I.P. in a volume of approximately 0.5 ml of sterile saline, using a sterile 25-gauge needle or through an Alzets pump. Optionally, the solution comprising the antibacterial oligonucleotide may also be administered I.V., subcutaneously, orally, or by any other means suitable for the given pathogen being tested.

Where applicable, bacteremia will be monitored by collecting daily blood samples from two animals from each group. One fully-anesthetized animal from the negative control group (no bacterial infection) will be bled by cardiac puncture and subsequently euthanized. The number of colony forming units (CFU) in the blood samples will then be determined by plating samples on agar and doing bacterial colony assays.

The minimum lethal dose for a given bacterial pathogen, *e.g., Escherichia coli* ATCC accession No. 25922, is determined for CFI mice after the pathogen is injected I.P. in 0.5 ml DPBS or S.C. plus agar. The minimum lethal inoculum is the minimum dose that results in the death of all of the test subjects during the five to seven days post-infection.

Alternatively, female NMRJ mice may be used with, for example, *Escherichia coli* ATCC accession No. 25922, which is known to cause animal death within five to seven days after intra-clavicular injection.

The dose of antibacterial oligonucleotide that protects 50% of the test animals from death (protective doses 50%-PD_{50})

is determined as follows. Beginning at various times after injection of the bacterium into the test animals, and continuing for four days thereafter, the antibacterial oligonucleotide (or its control) is injected S.C. into the test animals in about 0.15 ml DPBS at final concentrations that will vary as appropriate for the given assay. For example, about 0.0, 1.0, 2.0, 2.5, and 5.0 mg/kg of antibacterial oligonucleotide may typically be used. Animals surviving for more than five to seven days after initial bacterial inoculation will be maintained an additional seven days, and then euthanized by CO_2 asphyxiation for further study. Optionally, the test animals are maintained for more extended periods after initial infection in order to assess the long-term efficacy of oligonucleotide treatment.

A similar bacterial inoculation and oligonucleotide treatment protocol can be used to determine the kinetics of bacteria clearance from the peripheral blood of bacteremic animals after treatment with antibacterial oligonucleotide. In these studies, groups of twelve animals each are infected as above with *Escherichia coli*, and a group of six mice is sham injected with only saline (the control group). The groups of infected mice are then treated with (a) saline or (b) oligonucleotide, while the control group is only treated with saline. At suitable time periods post-infection, blood samples are taken, and the number of test pathogen cells per ml of blood is determined by standard dilution and culture methods.

The above animal models are merely exemplary of the myriad of animal models that may be used to establish the efficacy of the presently described antibacterial oligonucleotides, and many other modalities for testing the claimed invention are available to one of ordinary skill. For example, the LD_{50} of a given pathogen may be established (or previously known), and the efficacy of the antibacterial oligonucleotide determined, testing whether substantially all of the test animals survive bacterial exposure.

Additionally, immunocompromised animals may also be used, *i.e.*, nude mice, SCID mice, etc., to study the antibacterial effects of the described oligonucleotides in the absence of a correctly functioning immune system.

4.7. Pharmaceutical Compositions And Delivery

Pharmaceutical compositions containing the oligonucleotides of the invention in intimate admixture with a pharmaceutical carrier can be prepared according to conventional pharmaceutical compounding techniques. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., intravenous, oral, topical, aerosol (for topical or inhalation therapy), suppository, parenteral, or spinal injection.

In preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed, such as, for example, water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents and the like in the case of oral liquid preparations (such as, for example, suspensions, elixirs, and solutions); or carriers such as starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations (such as, for example, powders, capsules and tablets). Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. If desired, tablets may be sugar-coated or enteric-coated by standard techniques. Oral dosage forms of antibacterial oligonucleotides will be particularly useful for the treatment of bacterial infections of the gastrointestinal tract and ulcers caused by or associated with bacterial infection (*e.g.*, *Helicobacter pylori* infection, and the like). Additionally, given that bacterial infection has been associated with hyperproliferative disorders of the immune system (i.e. inflammatory bowel disease), the presently

described antibacterial oligonucleotides may be used to treat hyperproliferative disorders including, but not limited to, Crohn's disease and ulcerative colitis by specifically eliminating the causative or contributory microorganisms from the bacterial flora of the gut.

For parenteral application by injection, preparations may comprise an aqueous solution of a water soluble, or solubilized, and pharmaceutically acceptable form of the antibacterial oligonucleotide in an appropriately buffered saline solution. Injectable suspensions may also be prepared using appropriate liquid carriers, suspending agents, pH adjusting agents, isotonicity adjusting agents, preserving agents, and the like may be employed. Actual methods for preparing parenterally administrable compositions and adjustments necessary for administration to subjects will be known or apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Science, 15th Ed., Mack Publishing Company, Easton, Pa (1980), which is incorporated herein by reference. The presently described oligonucleotides should be parenterally administered at concentrations below the maximal tolerable dose (MTD) established for the antibacterial oligonucleotide.

For topical administration, the carrier may take a wide variety of forms depending on the preparation, which may be a cream, dressing, gel, lotion, ointment, or liquid.

Aerosols are prepared by dissolving or suspending the oligonucleotide in a propellant such as ethyl alcohol or in propellant and solvent phases. The pharmaceutical compositions for topical or aerosol form will generally contain from about 0.01% by weight (of the oligonucleotide) to about 40% by weight, preferably about 0.02% to about 10% by weight, and more preferably about 0.05% to about 5% by weight depending on the particular form employed.

Suppositories are prepared by mixing the oligonucleotide with a lipid vehicle such as theobroma oil, cacao butter, glycerin, gelatin, or polyoxyethylene glycols.

The presently described antibacterial oligonucleotides may be administered to the body by virtually any means used to administer conventional antibiotics. A variety of delivery systems are well known in the art for delivering bioactive compounds to bacteria in an animal. These systems include, but are not limited to, intravenous or intramuscular or intrathecal injection, nasal spray, aerosols for inhalation, and oral or suppository administration. The specific delivery system used depends on the location of the bacteria, and it is well within the skill of one in the art to determine the location of the bacteria and to select an appropriate delivery system.

The present invention is further illustrated by the following examples, which are not intended to be limiting in any way whatsoever.

5.0. EXAMPLES

5.1. Oligonucleotide Synthesis

Oligonucleotides were synthesized using commercial phosphoramidites on commercially purchased DNA synthesizers at either 1 μM or 15 μM scales using standard phosphoramidite chemistry. Oligonucleotides were deprotected following phosphoramidite manufacturers protocols. Oligonucleotides to be used unpurified were either dried down under vacuum or precipitated and then dried.

Sodium salts of oligonucleotides were prepared using the commercially available DNA-Mate (Barrskogen, Inc.) reagents or conventional techniques such as the commercially available exchange resin, *e.g.*, Dowex (Tradename), or by addition of sodium salts followed by precipitation, diafiltration, or gel filtration,. etc.

Oligonucleotide preparations that would be subject to further purification were initially chromatographed on commercially available reverse phase or ion exchange media (preferably, SAX, strong anion exchange media) such as Source Q made by Pharmacia, Toyopearl super Q made by Tosohaas, Protein Pak made by Waters, Macroprep Q made by BioRad, and

the like. Peak fractions were combined and the samples desalted and concentrated by ethanol precipitation, diafiltration, or gel filtration followed by lyophilization or solvent evaporation under vacuum in commercially available instrumentation such as Savant's Speed Vac. Optionally, the oligonucleotides may also be electrophoretically purified using polyacrylamide gels.

A variety of commercially available gel filtration media are particularly well suited for the desalting and/or purification of antibacterial oligonucleotides. Gel filtration media which may be used include Sephadex or Superdex made by Pharmacia, Trisacryl made by BioSepra, BioGel (preferably P-series, or more preferably P4) made by BioRad, Toyopearl HW SEC made by Tosohaas, Cellufine made by Amicon, and the like. Optionally, the gel filtration step may be repeated several times in order to better remove low molecular weight species, and particularly alkyl amines and/or alkyl ammonium compounds, from the oligonucleotide preparations.

Cation exchange columns comprising media such as Macroprep S (or CM) made by BioRad (preferably in the NH_4^+ form), Dowex resins, or Amberlite resins are also useful to remove contaminants from antibacterial oligonucleotide preparations. Typically, the pH of the eluted oligonucleotide will be increased to about 7-8 using ammonium hydroxide consequential to this step.

Alternatively, exhaustive dialysis or diafiltration may be used to remove salts or contaminants that inhibit or mask the antibacterial activity of the oligonucleotides (e.g., alkyl amines and/or alkyl ammonium compounds). Exhaustive butanol extractions, chloroform extraction followed by ethanol washes or multiple ethanol extractions may be used to obtain purified oligonucleotides that retain antibacterial activity.

Oligonucleotides to be used in bacterial experiments were dissolved in pyrogen free, sterile, physiological saline (*i.e.*, 0.85% saline), sterile Sigma H_2O, and filtered through

a 0.45 micron Gelman filter (or a sterile 0.2 micron pyrogen free filter prior to animal studies). Table 1 contains a list of all oligonucleotide sequences used in the examples. Although the majority of oligonucleotides used in the examples were constructed using a phosphorothioate backbone, unless otherwise noted, it should be understood that any of a wide variety of chemical backbones could be also used to generate oligonucleotides comprising the sequences listed in Table 1. The antibacterial oligonucleotides were tested for inhibition (INH) activity against drug resistant Gram negative (*Escherichia coli* ATCC accession No. 35218) and Gram positive (*Staphylococcus aureus* ATCC accession No. 13301) microorganisms. The percent inhibition data in Table 1 were averaged and normalized to a concentration of 2 mg/ml.

Tables 2(A-W) provide time course experiments that test the inhibitory activity (against *Escherichia coli* ATCC accession No. 35218 or *Staphylococcus aureus* ATCC accession No. 13301) of the indicated oligonucleotides when present at 2 mg/ml in the culture medium as targeted against genes that represent nearly all known gene classes in bacteria. In brief, Table 2A shows the inhibitory effect of oligonucleotide 28 (NBT 28, SEQ ID NO. 1); Table 2B tests oligonucleotide 10 (SEQ ID NO. 17); Table 2C tests oligonucleotide 43 (SEQ ID NO. 34), Table 2D shows the inhibitory effect of oligonucleotide 27 (SEQ ID NO. 45); Table 2E tests oligonucleotide 2 (SEQ ID NO. 120); Table 2F tests oligonucleotide 89 (SEQ ID NO. 61); Table 2G tests oligonucleotide 103 (SEQ ID NO. 64); Table 2H tests oligonucleotide 132 (SEQ ID NO. 65), Table 2I shows the inhibitory effect of oligonucleotide 19 (SEQ ID NO. 66); Table 2J tests oligonucleotide 16 (SEQ ID NO. 72); Table 2K tests oligonucleotide 96 (SEQ ID NO. 79); Table 2L tests oligonucleotide 21 (SEQ ID NO. 85); Table 2M shows the inhibitory effect of oligonucleotide 18 (SEQ ID NO. 95); Table 2N tests oligonucleotide 105 (SEQ ID NO. 103); Table 2O tests oligonucleotide 46 (SEQ ID NO. 105); Table 2P tests oligonucleotide 114 (SEQ ID NO. 112); Table 2Q tests

oligonucleotide 32 (SEQ ID NO. 116); Table 2R tests oligonucleotide 73 (SEQ ID NO. 124); Table 2S tests oligonucleotide 63 (SEQ ID NO. 130), Table 2T shows the inhibitory effect of oligonucleotide 78 (SEQ ID NO. 134); Table 2U tests oligonucleotide 71 (SEQ ID NO. 151); Table 2V tests oligonucleotide 14 (SEQ ID NO. 154); and Table 2W tests oligonucleotide 5 (SEQ ID NO. 152).

5.2. MIC With *Escherichia coli*

Oligonucleotides from every known gene class in bacteria were used to test inhibition of bacterial growth in a modified MIC test (described above). In all cases the control bacterial cells entered exponential growth while the test cells to which oligonucleotide had been added showed no growth at all or significant inhibition of growth (see Table 1).

Similar results were achieved with other oligonucleotides selected using the parameters described above, which were subsequently synthesized, purified and tested using the same MIC analysis. See Table 1.

The results in Table 1 demonstrate that antisense or antigene (inhibition of expression by DNA triplex formation) oligonucleotides are effective against a variety of genes. For example: genes involved in energy metabolism (sugar metabolism, fatty acid metabolism), cell division (DNA replication, cell wall biosynthesis), global regulatory proteins, protein synthesis (tRNA synthesis, mRNA stability, rRNA synthesis, ribosomal protein, translation factors), virulence factors, cell wall and membrane synthesis (fatty acid and phospholipid synthesis, lipopolysaccharide synthesis, periplasmic-secretory proteins, transport proteins, outer-membrane proteins), amino acid biosynthesis, nucleic acid synthesis, nitrate reductase, vitamin metabolism, and drug resistance.

In fact, Figure 2 shows that the described antibacterial oligonucleotides proved effective against a wide variety of genes from both Gram negative and Gram positive bacteria.

More specifically, oligonucleotides targeted against bacterial genes relating to: energy metabolism (A); DNA replication (B); cell division (C); regulatory proteins (D); cell wall biosynthesis (E); sugar metabolism (F); virulence, pili, flagella (G); fatty acid metabolism (H); mRNA synthesis (I); tRNA synthesis (J); rRNA synthesis (K); ribosomal protein synthesis (L); protein synthesis (M); phospholipid synthesis (N); periplasmic/secretory protein synthesis (O); regulation and synthesis of transport proteins (P); amino acid biosynthesis and metabolism (Q); lipopolysaccharide synthesis (R); purine/pyrimidine biosynthesis and metabolism (S); outer membrane protein synthesis and regulation (T); nitrate reductase synthesis and regulation (U); drug resistance (V); and vitamin metabolism and biosynthesis (W) were capable of significantly inhibiting the growth of both Gram negative and Gram positive bacteria.

Thus, antibacterial oligonucleotides were effective against virtually every major cellular function tested (as determined by the MIC assay).

As additional genome sequence data are obtained for bacteria, this invention may be extended to oligonucleotide targets within newly described bacterial sequences. Antibacterial oligonucleotides may be constructed with a range of backbones including, but not limited to: phosphorothioates; p-ethoxy oligonucleotides (partially or fully substituted); or 2'-O-methyl oligonucleotides (partially or fully substituted). Oligonucleotides comprising all of the above backbones have proved equally effective in inhibiting bacterial growth. In view of the effectiveness of oligonucleotides comprising the chemical backbones listed above, chimeric oligonucleotides (comprising mixed backbones) are also deemed to be effective antibacterial agents.

Several oligonucleotides based on the NBT 18 sequence (SEQ ID NO. 95) were also capable of inhibiting the growth of two clinically relevant pathogens that have proven resistant to most conventional antibiotics - *Escherichia coli* clinical

isolate ATCC accession No. 35218 (Tables 3A and 3B), and *Staphylococcus aureus* clinical isolate ATCC accession No. 13301 (Tables 3C and 3D). The NBT 18 sequence variations that were tested in Tables 3A and 3B include: A - the NBT 18 sequence with a 2'-O-Methoxy substituted backbone; B - a truncated (12mer, SEQ ID NO. 174) version of the NBT 18 sequence with a phosphorothioate backbone; C - a truncated (15mer, SEQ ID NO. 175) region of the NBT 18 sequence with a phosphorothioate backbone; D - a truncated (15mer) region of the NBT 18 sequence with a phosphorothioate backbone and a 5' amino group; and E - the NBT 18 sequence with a phosphorothioate backbone. The NBT 18 sequence variations that were tested in Tables 3C and 3D include: A - the NBT 18 sequence with a 2'-O-Methoxy substituted backbone; B - the NBT 18 sequence with a p-ethoxy substituted backbone; C - a truncated (12mer) region of the NBT 18 sequence with a phosphorothioate backbone; D - a truncated (15mer) region of the NBT 18 sequence with a phosphorothioate backbone; and E - a truncated (18mer, SEQ ID NO. 176) region of the NBT 18 sequence with a phosphorothioate backbone. The data in Tables 3(A-D) indicate that the observed antibacterial effect was largely a feature of the antisense sequence of NBT 18 instead of the backbone of a given oligonucleotide (*i.e.*, nonspecific sulphur effects, etc.).

These data further indicate that oligonucleotides comprising less than one half of the full-length (27 base) sequence of NBT 18 retain the ability to inhibit the growth of at least two clinically significant pathogens.

5.3. <u>MIC With Gram Negative And Gram Positive Bacteria</u>

A representative number of the antisense oligonucleotides were tested against a wide variety of bacterial species including *Streptococcus* (*Streptococcus mutans* (ATCC accession No. 25175)), *Streptococcus pyogenes* (ATCC accession No. 14289), *Streptococcus pneumoniae* or *Pneumococcus pneumoniae* (ATCC accession No. 39937), and *Streptococcus faecalis* or *Enterococcus faecalis* (ATCC

accession No. 19433), *Staphylococcus aureus* (ATCC accession No. 29213), *Staphylococcus aureus* (ATCC accession No. 13301), *Escherichia coli* (ATCC accession Nos. 11370, 25922, and 29214), *Salmonella typhimurium* (ATCC accession No. 23564), *Pseudomonas fluorescens* (ATCC accession No. 13525), *Klebsiella pneumoniae* (ATCC accession No. 4352), *Serratia liquefaciens* (ATCC accession No. 27592), *Neisseria sicca* (ATCC accession No. 9913), *Mycobacterium smegmatis* (ATCC accession No. 19420), *Yersinia mollareti* (ATCC accession No. 43969), *Haemophilus segnis* (ATCC accession No. 33393), *Haemophilus vaginalis* (ATCC accession No. 14018), *Shigella* sp. (ATCC accession No. 11126), *Vibrio fischeri* (ATCC accession No. 7744), and *Helicobacter mustelae* (ATCC accession No. 43772).

Representative data generated with phosphorothioate forms of the oligonucleotides are provided in Tables 4(A-Z). In brief, antibacterial oligonucleotides nos. 18 (SEQ ID NO. 73), 39 (SEQ ID NO. 30), 63 (SEQ ID NO. 130), 78 (SEQ ID NO. 134), and 73 (SEQ ID NO. 124) were tested against *Salmonella typhimurium* (Tables 4A and 4B); antibacterial oligonucleotides 39 (SEQ ID NO. 30), 63 (SEQ ID NO. 130), 78 (SEQ ID NO. 134), 82 (SEQ ID NO. 161), and 114 (SEQ ID NO. 112) were tested against *Pseudomonas aeruginosa* (Tables 4C and 4D); antibacterial oligonucleotides 114 (SEQ ID NO. 112), 78 (SEQ ID NO. 134), 73 (SEQ ID NO. 124), 71 (SEQ ID NO. 151), and 111 (SEQ ID NO. 132) were tested against *Klebsiella pneumoniae* (Tables 4E and 4F); antibacterial oligonucleotides 2 (SEQ ID NO. 50), 4 (SEQ ID NO. 173), 127 (SEQ ID NO. 143), 63 (SEQ ID NO. 130), and 73 (SEQ ID NO. 124) were tested against *Yersinia mollaretti* (Tables 4G and 4H); antibacterial oligonucleotides 16 (SEQ ID NO. 72), 12 (SEQ ID NO. 80), 20 (SEQ ID NO. 84), 3 (SEQ ID NO. 121), and 15 (SEQ ID NO. 81) were tested against *Neisseria sicca* (Tables 4I and 4J); antibacterial oligonucleotides 2 (SEQ ID NO. 50), 39 (SEQ ID NO. 30), 82 (SEQ ID NO. 161), and 114 (SEQ ID NO. 112) were tested against *Serratia liquefaciens* (Table 4K); antibacterial oligonucleotides 1 (SEQ ID NO. 119), 89 (SEQ ID

NO. 61), 127 (SEQ ID NO. 143), 132 (SEQ ID NO. 15), and 114 (SEQ ID NO. 112) were tested against *Streptococcus mutans* (Tables 4L and 4M); antibacterial oligonucleotides 1 (SEQ ID NO. 119), 89 (SEQ ID NO. 61), 127 (SEQ ID NO. 143), 132 (SEQ ID NO. 15), and 114 (SEQ ID NO. 112) were tested against *Streptococcus pyogenes* (Tables 4N and 4O); antibacterial oligonucleotides 1 (SEQ ID NO. 119), 89 (SEQ ID NO. 61), 127 (SEQ ID NO. 143), 132 (SEQ ID NO. 15), and 114 (SEQ ID NO. 112) were tested against *Shigella* (Tables 4P and 4Q); antibacterial oligonucleotide 78 (SEQ ID NO. 134) was tested against *Haemophilus* (Table 4R); antibacterial oligonucleotides 114 (SEQ ID NO. 112), 10 (SEQ ID NO. 17), 21 (SEQ ID NO. 85), 18 (SEQ ID NO. 73), and 78 (SEQ ID NO. 134) were tested against *Mycobacterium* (Tables 4S and 4T); antibacterial oligonucleotide 78 (SEQ ID NO. 134) was tested against *Helicobacter* (Table 4U); antibacterial oligonucleotides 89 (SEQ ID NO. 61), 127 (SEQ ID NO. 143), 132 (SEQ ID NO. 15), p127 (SEQ ID NO. 143 with a p-Ethoxy backbone), 1 (SEQ ID NO. 119), and 76 (SEQ ID NO. 127) were tested against *Enterococcus* (Tables 4V and 4W); antibacterial oligonucleotides 1 (SEQ ID NO. 119), 78 (SEQ ID NO. 134), 114 (SEQ ID NO. 112), 127 (SEQ ID NO. 143), and 132 (SEQ ID NO. 15) were tested against *Streptococcus pneumonia* (Tables 4X and 4Y); and antibacterial oligonucleotides 78 (SEQ ID NO. 134) and 127 (SEQ ID NO. 143) were tested against *Vibrio* (Table 4Z). The data in Tables 4A-Z indicate that the antibacterial oligonucleotides targeted to varying classes of genes are capable of strongly inhibiting the growth of a broad spectrum of bacterial species. No significant difference in antibacterial activity was found when different stereoisomers of phosphorothioate backbone oligonucleotides were tested.

Additionally, Figures 3(a-c) respectively provide time course data providing percent inhibition as a function of time for oligonucleotides 73 (SEQ ID NO. 124), 63 (SEQ ID NO. 130), and 18 (SEQ ID NO. 73) as measured against *Salmonella typhimurium*; Figures 4(a-c) respectively provide time course

data showing percent inhibition as a function of time for oligonucleotides 39 (SEQ ID NO. 30), 78 (SEQ ID NO. 134), and 63 (SEQ ID NO. 130) as measured against *Pseudomonas aeruginosa;* and Figures 5(a-b) respectively provide time course data showing percent inhibition as a function of time for oligonucleotides 73 (SEQ ID NO. 124) and 114 (SEQ ID NO. 112) as measured against *Klebsiella pneumoniae*.

In view of the wide range of bacteria already successfully tested, any oligonucleotides chosen and prepared in the manner described herein will be equally effective against a given bacterial target. In addition to the species explicitly mentioned herein, a wide variety of other bacterial pathogens may be treated using the described compositions. A relatively comprehensive review of such pathogens is provided, *inter alia*, in Mandell *et al.*, 1990, Principles and Practice of Infectious Disease 3rd. ed., Churchill Livingstone Inc., New York, N.Y. 10036, herein incorporated by reference.

5.4. **MIC At 24 Hours**

In order to distinguish whether the antibacterial oligonucleotides had transient bacteriostatic effects, or long lasting effects, MIC assays were extended to include a time point of over 24 hours. These data are presented in Tables 5A-D. Tables 5A and 5B show, *inter alia*, that oligonucleotides 21 (SEQ ID NO. 156), 68 (SEQ ID NO. 148), and 85 (SEQ ID NO. 106), 112 (SEQ ID NO. 62), and 18 (SEQ ID NO. 73) continue to substantially inhibit the growth of *Staphylococcus aureus* ATCC accession No. 13301, for at least 25 hours. These data indicate that the tested oligonucleotides have long-term bacteriostatic or bactericidal (see Figure 9, below) effects on *Staphylococcus aureus* ATCC accession No. 13301. Moreover, the timing of antibacterial oligonucleotide addition does not significantly affect the observed antibacterial activity since activity was seen when the addition of antibacterial oligonucleotide was delayed for 180, 350, or 480 min.

Conversely, Tables 5C-D indicates that, although a substantial amount of growth inhibition occurs initially, the same oligonucleotides do not significantly inhibit the growth of *Escherichia coli* ATCC accession No. 35218 when growth was assayed 27 hours after the bacteria were initially exposed to the oligonucleotides. The data in Tables 5C and 5D indicate that oligonucleotides 21 (SEQ ID NO. 156), 68 (SEQ ID NO. 148), 85 (SEQ ID NO. 106), 112 (SEQ ID NO. 62), and 18 (SEQ ID NO. 73) are bacteriostatic for *Escherichia coli* ATCC accession No. 35218. *Escherichia coli* ATCC accession No. 35218 represents a particularly virulent, multiple drug resistant strain of *Escherichia coli*. When oligonucleotide number 89 (SEQ ID NO. 61) was tested against *Escherichia coli* accession No. 25922, a moderately penicillin resistant strain, a dose-dependent long lasting bacteriostatic effect was observed (see Tables 5E and 5F). It is expected that multiple doses of the same oligonucleotide, rather than a single dose, might result in enhanced long-term activity against the more resistant *Escherichia coli* ATCC accession No. 35218.

The 24-hour MIC studies were performed essentially as described above with the exceptions that: growth of the target bacteria to reach an OD_{525} of 0.1 occurs in approximately 8 hours instead of about 12 to 16 hours; bacterial growth is monitored throughout the experiment as well as at the end-points; and an additional test was conducted that used starved cells as the initial inoculum instead of fresh log cultures (which provided similar antibacterial results).

5.5. Purification Studies

The MIC test was carried out as described in Section 4.5., *supra*. The test oligonucleotides received various post-synthesis treatments, and the percent inhibition of the cell culture growth was calculated as described *supra*. See Tables 6A and 6B.

Oligonucleotide NBT 78 (SEQ ID NO. 134), was given the following treatments:

A. butanol precipitated and resuspended as an ammonium salt;

B. butanol precipitated, converted to a sodium salt, desalted on a gel filtration column (described Section 5.1);

C. purified via anion exchange HPLC, desalted by gel filtration;

D. butanol precipitated, converted to a sodium salt, desalted on a reverse phase HPLC column (trityl off);

E. butanol precipitated, ammonium hydroxide added, desalted via gel filtration, left as an ammonium salt;

F. butanol precipitated once, filtered through a 0.45 micron filter (e.g., Gelman Acrodisc, Millipore, Nalgene, etc.) followed by ethanol precipitation;

G. butanol precipitated twice, filtered through a 0.45 micron filter (e.g., Gelman Acrodisc, Millipore, Nalgene, etc.), and washed three times with 95% ethanol;

H. butanol precipitated twice, filtered through a 0.45 micron filter (e.g., Gelman Acrodisc, Millipore, Nalgene, etc.), washed with chloroform and ethanol;

I. butanol precipitated twice, filtered through a 0.45 micron filter (e.g., Gelman Acrodisc, Millipore, Nalgene, etc.), butanol precipitated 2 more times, and washed once with ethanol.

The results in Tables 6A and 6B demonstrate that the protocol used to purify the oligonucleotides greatly affects bacterial susceptibility in a MIC test. Oligonucleotides that are treated only by butanol precipitation inhibited bacterial growth by less than 25 percent. However, oligonucleotides that were subject to: a) gel filtration; b) four butanol precipitations; or c) two butanol extractions, followed by ethanol or chloroform extractions all

demonstrated greater than 85% inhibition of the growth of the test bacteria used in the MIC assay (see B, C, E, G, H and I). Oligonucleotides may also be purified by strong anion exchange (SAX) chromatography, reverse-phase chromatography, strong cation exchange (SCX) chromatography, followed by size exclusion chromatography (SEC). Alternatively, after the first SCX column, a second SCX column can be run followed by a reverse-phase chromatography step. Optionally, the SCX step may be supplemented or replaced by an alcohol (e.g., ethanol, etc.) precipitation step.

The above results demonstrated that proper post synthesis handling protocols play an integral role in the production of oligonucleotides that display antibacterial activity.

There are a variety of contaminants that may be present in an oligonucleotide preparation after cleavage from the solid supports and removal of the protecting groups, and even after HPLC treatment. These contaminants include residual protecting groups, and contaminants that are introduced or generated during synthesis or purification. Examples of such contaminant include, but are not limited to, quaternary amines (particularly alkyl amines and/or alkyl ammonium compounds), acetamide, acetic acid, 2-cyanoethanol, isobutyramide, isobutyric acid, benzamide, benzoic acid, succinimide, succinic acid, t-butylphenoxyacetamide (or acetic acid), phenoxyacetamide (or acetic acid). Given the results shown in Tables 6A and 6B, it is clear that the substantial removal of the above or other contaminants greatly enhances the antibacterial activity of an oligonucleotide.

Contaminants that are particularly important to remove from the oligonucleotide preparations include compounds that directly or indirectly inhibit bacterial uptake of the oligonucleotides, or otherwise mask the antibacterial effects of the oligonucleotides. One way that a contaminant may mask the antibacterial efficacy of an oligonucleotide is by stimulating bacterial growth in a manner that effectively

compensates for the antibacterial activity of a given oligonucleotide. Accordingly, the present finding that certain contaminants (i.e., alkyl amines and/or alkyl ammonium compounds) that are typically present in conventional oligonucleotide preparations may mask the *in vitro* antibacterial activity of oligonucleotides represents a seminal discovery that requires a fundamental reassessment of the utility of oligonucleotides as antibacterial agents *in vivo*.

In particular, an impurity in anion exchange (AX) HPLC-purified modified linkage oligonucleotides has been isolated and partially characterized which stimulates bacterial growth both *in vitro* and *in vivo*. This impurity/stimulatory material is a mixture of small, polar, multialkyl amino or alkyl ammonium compounds that have negligible absorbance at 254 nm. The impurity is apparently generated from the AX-HPLC stationary phase during the elution gradient.

The absence of an active chromophore at 254 nm effectively renders the impurity invisible to the absorbance detectors used during HPLC of DNA oligonucleotides. Since anion exchange chromatography precludes the use of conductivity detectors to monitor peaks, the impurity is also virtually invisible during the purification and analytical HPLC procedures typically used in the manufacture of oligonucleotides.

As shown above, the impurity can be removed and isolated from the oligonucleotide preparations by using a series of desalting steps. For example, in the first step, the oligonucleotide was concentrated by first loading the pooled fractions of an AX purification run onto appropriately sized Hamilton PRP-1 or PRP-3 columns. The salt was then removed from the column by washing with water until the conductivity of the wash eluant was below 25 μS/cm. Finally, the oligonucleotide was eluted as a concentrated solution (app. 100-300 OD's per mL) using a moderately steep (5% per minute) gradient of water:90% ethanol. It should also be noted that oligonucleotides purified in this manner must contain at

least two phosphorothioate or p-ethoxy linkages, or some other non-polar modification in order to adequately absorb to the stationary phase.

In the second step, the oligonucleotide solution was concentrated or removed entirely by lyophilization prior to further purification by size-exclusion chromatography (SEC). The oligonucleotide was re-suspended in a minimum amount of water prior to application to the SEC column. Since essentially all of the salt from the AX purification was removed by the RP step, the oligonucleotide was dissolved in a relatively small volume of water. This small volume helps maximize resolution in the SEC step.

A column was prepared using virgin BioGel P-4 medium or fine particle SEC medium, using a modified manufacturer's procedure to swell the medium. The column used was 45-50 cm long and 2.2 cm diameter. The flow rate was approximately 1-2 mL/minute. This size column can be used to purify 1,000-3,000 OD's of modified linkage oligonucleotides that are at least 12 bases in length. If the oligonucleotides have more than 30% phosphorothioate linkages, the maximum loading drops to about 2,000 OD's. Columns and sample sizes may be scaled up as long as a flow velocity of about 30-75 cm/hr is maintained, and the column height remains at least about 40 cm.

The oligonucleotides were eluted with water while monitoring the conductivity and the absorbance at 254 nm. The purification may be easily be modified by monitoring at 280 nm, and the like. Collection began when the oligonucleotide concentration became appreciable (as measured by O.D.), and stopped at no later than about 8 minutes after collection began. If, after the conductivity initially rose, it fell and then began to rise again, collection was terminated. It was important to stop collection as described because oligonucleotides collected after this point typically included the stimulatory impurities.

The collected oligonucleotide solutions were checked for concentration and lyophilized. Typically, the above protocol

resulted in the purified oligonucleotides having the desired antimicrobial activities.

When separation continued after the collection of the oligonucleotide peak, several other peaks were seen which displayed little to no absorbance at 254 nm, but noticeable conductivity. The amount of impurity observed varied for each individual purification. The variation was probably attributable to the different salt concentrations required to elute different oligonucleotides, or variations in the length of time since the AX column was last used, etc.

While the detected amounts of impurity generally remained a small percentage of the net composition, both *in vivo* and *in vitro* testing showed that the impurities stimulate bacterial growth. Oligonucleotides that were not purified by AX-HPLC but are otherwise treated the same did not display either of the peaks observed during SEC, and did not have a stimulatory effect. However, oligonucleotides that were AX-HPLC purified and desalted as described, but were not further purified by SEC showed either stimulatory effects or, where the amounts of the impurities were not high, neutral or a significantly reduced antibiotic effect.

Spectroscopic analysis (^{1}H-NMR, A_{254} absorbance, GC-MS, and FAB and ESI positive ion mass spectrometry) pointed to a comparatively small, simple molecule, or mixture of similar components, that were eluted along with the oligo. These compound(s) coeluted with oligonucleotide during the reverse-phase concentration/desalting process. In particular, analysis by electrospray mass spectroscopy of small molecular weight material removed from an oligonucleotide preparation that had been purified on a Waters Protein Pak 40Q revealed complex mixture of amino compounds with the common feature of signals at m/z 58 and m/z 72. These two signals are derived from the N,N-diethyl-N-(2-hydroxypropyl) quarternary amino functional group used as the cationic absorption moiety on the Protein Pak Q SAX stationary phase. Electrospray analysis of similar material from a N,N,N-trimethyl quarternary amino polymer-based SAX phase (e.g., BioRad's

Macroprep Q) also yielded equivalent signals indicative of the cleavage of absorption sites from the stationary phase. These low molecular weight materials were removed by SEC, and were also removed by a combination of SCX and reverse phase chromatography.

The steep ramping required for concentration purposes did not permit conditions suitable for resolution of close-running materials. However, the SEC step outlined above was capable of sufficiently removing the impurities to allow the detection of a consistent pattern of antibiotic activity inherent in the presently described purified oligonucleotides. Accordingly, the SEC step provides a process that allowed for the consistent and predictable removal of the stimulatory impurities from the oligonucleotide preparations.

As discussed above, oligonucleotides that have been purified using different procedures (i.e., no chromatography steps) consistently showed antibiotic effects that were comparable to the oligonucleotides purified as outlined immediately above.

In some very non-polar oligonucleotides, such as total p-ethoxy and chimeras with p-ethoxy/2'-O-methyl RNAs components, the concentration of ethanol required to elute the oligonucleotides from the reverse-phase column was high enough to allow some removal of the low-absorbing high conductivity material prior to the elution of the oligonucleotides. However, the resolution was not sufficiently clean to allow straight-forward characterization. This separation was not observed with predominantly S-oligonucleotides.

The ability of the RP-column to provide any separation may also be affected by the base composition of the oligonucleotides as well as the type of linkages employed to construct the oligonucleotides. Typically, the use of ethanol provided more control over the elution process than acetonitrile, which has higher elution power than ethanol.

Additionally, the use of ethanol during this step has implications for cGMP validation.

Another feature of the RP step is that the great reduction of inorganic salt during the reverse-phase protocol allows for the use of conductivity to monitor peak elution during the SEC separation. If the salt were not removed, the conductivity signal of the impurities would be masked by the signal from the salt, and conductivity would only be useful for monitoring gross system changes.

The alkyl amines and/or alkyl ammonium compounds present in the described impurity apparently act as a counter ion to the phosphodiesters and/or associated to the polar portions of the triester groups of the antibacterial oligonucleotides. The impurity material can not be isolated from blank runs of solutions, reagents, and stationary phases used during the described synthesis and purification procedures. Presently, the impurity has only been observed in oligonucleotides that have been AX purified.

Further characterization (by spectroscopic analysis) of the stimulatory impurities isolated during the SEC step revealed that they are apparently produced by cleavage of absorption sites on the SAX stationary phase.

Although relatively crude oligonucleotide preparations were able to demonstrate significant inhibition in this assay (after substantial removal of the contaminants that normally hinder the antibacterial effects of oligonucleotides), FDA requirements for parenteral therapeutics necessitate higher levels of purification for animal and human use.

5.6. <u>Antigene Antibacterial Oligonucleotide Activity</u>

Antibacterial oligonucleotides 96ss (SEQ ID NO. 79) and 73ss (SEQ ID NO. 124) (the ss denotes that oligonucleotide 73 is targeted to the sense strand) are homologous to the sense strand of the targeted sequences. Oligonucleotides 96ss and 73ss are thought to exert antibacterial activity by acting as antigene sequences that block gene expression by forming a triple-stranded complex (*i.e.*, triplex formation), or,

possibly, by directly interacting with bacterial proteins. A time course of the antibacterial activity of oligonucleotides 73ss and 96ss is shown in Table 7.

5.7. The Use of Antibacterial Oligonucleotides Against Antibiotic Resistant Bacteria

The presently described antibacterial oligonucleotides are also capable of inhibiting the growth of a variety of bacteria that are known to be resistant to various traditional antibiotics. Tables 8(A-C and F) test the inhibitory activity of oligonucleotide 73 (NBT 73 - SEQ ID NO. 124) against clinical isolates of *Escherichia coli* that are known to be resistant to: streptomycin (8A); sulfonamide (8B); penicillin (8C); as well as multiple drug resistant *Escherichia coli* (8F). Oligonucleotide 114 (SEQ ID NO. 112) also inhibited the growth of *Salmonella typhimurium* ATCC accession No. 23564 (8D), *Klebsiella pneumoniae* ATCC accession No. 4352 (8E), and *Staphylococcus aureus* ATCC accession No. 29213 (8G).

Tables 9(A-G) test the inhibitory activity of oligonucleotide 114 (NBT 114 - SEQ ID NO. 112) against clinical isolates of *Escherichia coli* that are known to be resistant to: streptomycin (9A); sulfonamide (9B); penicillin (9C); as well as multiple drug resistant *Escherichia coli* (9F). Oligonucleotide 114 (SEQ ID NO. 112) also inhibited the growth of *Salmonella typhimurium* ATCC accession No. 23564 (9D), *Klebsiella pneumoniae* ATCC accession No. 4352 (9E), and *Staphylococcus aureus* ATCC accession No. 29213 (9G).

Additional studies revealed that antibacterial oligonucleotides 114 (SEQ ID NO. 112), 5 (SEQ ID NO. 152), 39 (SEQ ID NO. 30), 43 (SEQ ID NO. 34), 3 (SEQ ID NO. 51), 78 (SEQ ID NO. 134), 12 (SEQ ID NO. 153), 14 (SEQ ID NO. 154), 23 (SEQ ID NO. 158), 24 (SEQ ID NO. 159), 22 (SEQ ID NO. 157), 17 (SEQ ID NO. 83), 20 (SEQ ID NO. 84), 15 (SEQ ID NO. 81), 16 (SEQ ID NO. 82), 19 (SEQ ID NO. 66), 28 (SEQ ID NO. 96), 63 (SEQ ID NO. 130), 10 (SEQ ID NO. 17), and 18 (SEQ ID NO. 73) significantly inhibited the growth of multiple drug

resistant *Escherichia coli* ATCC accession No. 35218 for over 400 minutes when present at a concentration of about 0.5-2.0 mg/ml as shown in Figures 6(a-t).

Additionally, antibacterial oligonucleotides 16 (SEQ ID NO. 82), 18 (SEQ ID NO. 73), 1 (SEQ ID NO. 119), 5 (SEQ ID NO. 152), 17 (SEQ ID NO. 83), 21 (SEQ ID NO. 156), 132 (SEQ ID NO. 15), 11 (SEQ ID NO. 18), 89 (SEQ ID NO. 61), and 2 (SEQ ID NO. 50) all inhibited the growth of penicillin resistant clinical isolates of *Staphylococcus aureus* ATCC accession No. 13301 for over 400 minutes when present in the culture medium at a concentration of about 0.5-2.0 mg/ml (data are respectively provided in Figures 7(a-j)).

Oligonucleotide 14 (NBT 14 - SEQ ID NO. 154) was used to test whether the antibacterial oligonucleotides could also be used to enhance a target bacteria's sensitivity to antibiotics to which the bacteria had previously proven resistant. Table 10 shows the results of a growth inhibition time course experiment where oligonucleotide 14 was tested for the ability to inhibit the growth of *Escherichia coli* Y1088 (known to be resistant to ampicillin) in the presence and absence of the indicated concentration of ampicillin (50 μg/ml, and 250 μg/ml). Table 10 indicates that oligonucleotide 14 is capable of significantly restoring ampicillin sensitivity of *Escherichia coli* Y1088.

5.8. Animal Studies

Preliminary assessments of the *in vivo* efficacy of the presently described antibacterial oligonucleotides (using a Lister & Saunders test) indicate that a higher percentage of animals treated with oligonucleotide survive exposure to near-lethal amounts of *Escherichia coli* ATCC accession No. 25922 (prepared and injected as described in Lister & Saunders, 1995). In particular, Figure 8 shows that mice treated with oligonucleotide 114 (SEQ ID NO. 112) *in vivo* proved more resistant to challenge by a bacterial pathogen than control animals. The assay was conducted essentially as described in section 4.6, *supra*, and involved a total of 5 mg

of oligonucleotide injected (I.P.) over a 2 day period (1 mg of oligonucleotide suspended in 0.5 ml of sterile saline was injected at 1, 5, 10, 24, and 34 hours post infection). Additionally, Figure 9 shows that mice treated with the antibacterial oligonucleotide SOT 114.21 (phosphorothioate GGAACGCGC linked to 2'-methoxy riboCATTGGTATATC with an inverted 3' terminal deoxythymidine) had substantially enhanced survival after challenge with lethal quantities (approximately 10^8 cfu in mucin and iron dextran injected i.p. into CD1 mice) of *Staph. Aureus*. In Figure 9, treatment with *Staph.* was T=0 and 5 hours after infection. Oligonucleotide treatment was only administered on day 1.

Subsequent *in vivo* studies have shown that SOT 114.21 can increase the survival of *Staph. Aureus* challenged test animals by about 81 percent, and increase the survival of *E. coli* infected test animals by about 95 percent (relative to animals treated with a placebo).

Similarly, when a representative antibacterial oligonucleotide was tested using the model of Hof *et al.*, additional evidence of *in vivo* efficacy was obtained. In particular, Table 11 shows that mice treated with oligonucleotide 132 (SEQ ID NO. 15) *in vivo* had markedly reduced amounts of bacteremia 24 hours after initial exposure to *Escherichia coli* ATCC accession No. 25922 (prepared and injected as described in Hof *et al.*, 1986). This assay was conducted essentially as described in section 4.6, and involved the injection of a total of 2 mg of oligonucleotide (1 mg injected at 6 and 10 hours post infection).

5.9. Standard MIC Assays

To eliminate the possibility that the observed antibacterial activity might be a function of the slightly modified version of the MIC used to generate the above data, antibacterial assays were conducted using the standard MIC assay. Given that 44 percent of all nosocomial infections are caused by *Staph. aureus*, *Streptococcus*, or *Pseudomonas*, these bacteria were used as targets for standard MIC assays.

In brief, the standard MIC assay was conducted by using 10x13 mm tubes to which 40 μl of Mueller Hinton Broth (purchased from BBL, obtained through VWR, 3745 Bayshore Blvd., Brisbane, CA 94005) was added. The oligonucleotides (including an oligo dT control) were supplied as lyophilized pellets and dissolved in 200 μl of sterile tissue culture water (Sigma), and 200 μl aliquots of water or dissolved oligonucleotide were then added to the "control" or "oligo test" tubes.

Bacterial suspensions were prepared by suspending the organisms in 1.0 ml of sterile-filtered saline (Sigma) at a concentration corresponding to an $O.D._{625}$ of 0.1-0.102. Ten μl of this suspension was then added to 990 μl of saline and 500 ul of this mixture was added to both the "control" and "oligo test" tubes (a concentration of approximately $1x10^5$ bacteria per ml). Sterile saline was added (260 μl) to each of test tube to reach a total volume of 1 ml, the tubes were vortexed, $O.D._{625}$'s were measured (time zero), and tubes were incubated at 35° C for 16-24 hours (without shaking). Tubes were vortexed in the morning, and the amount of bacterial growth (if any) was measured by measuring $O.D._{625}$ readings.

Results from studies using the standard MIC assay are described in Figures 10 through 13.

The antibacterial oligonucleotides used in the following studies were constructed as follows (5' to 3'):

SOT T12, 12 thymidines (first six bases phosphorothioate deoxynucleotides, followed by six 2'-methoxy ribonucleotides and an inverted 3' terminal deoxythymidine linked by a 3'-3'

phosphodiester linkage); SOT-C12, 12 cytidines (first six bases phosphorothioate deoxynucleotides, followed by six 2'-methoxy ribonucleotides and an inverted 3' terminal deoxythymidine); SOT 89.6 (phosphorothioate deoxyCAT linked to 2'-methoxy riboGTC with an inverted 3' terminal deoxythymidine); SOT 89.9 (phosphorothioate deoxyCATGT linked to 2'-methoxy riboCATT with an inverted 3' terminal deoxythymidine); SOT 89.12 (phosphorothioate deoxyCATGTC linked to 2'-methoxy riboATTCTC with an inverted 3' terminal deoxythymidine); SOC 89.12 (phosphorothioate deoxyCATGTC linked to 2'-methoxy riboATTCTC with a 3' terminal cholesteryl group); SOB 89.12 (phosphorothioate deoxyCATGTC linked to 2'-methoxy riboATTCTC with a 3' terminal biotin group); MMT 89.12 (89.12 with all methoxyribonucleotides linked to an inverted 3' terminal deoxythymidine); MPT 89.12 (the 89.12 sequence, CATGTCATTCTC, with all p-ethoxy, 2'-methoxy RNA linked to an inverted 3' terminal deoxythymidine); SOPT 89.12 (phosphorothioate deoxyCATGTC linked to 2'-methoxy riboATTC followed by p-ethoxy, 2'-methoxy riboTC linked to an inverted 3' terminal deoxythymidine); POT 89.12 (89.12 with all p-ethoxy DNA linked to an inverted 3' terminal deoxythymidine); DSM 89.18 (phosphorothioate deoxyCATGTCAT linked to phosphorothio (i.e., sulphur), 2'-methoxyriboTCTCCTTAAG linked to a 3'-terminal deoxythymidine); SSM 89.18 (sulphur, 2'-methoxy riboCATGTCATTCTCCTTAAG linked to a 3'-terminal deoxythymidine); NBT 89.15 (phosphorothioate deoxy CATGTCATTCTCCTT linked to an inverted 3' terminal deoxythymidine); NBPT 89.12 (phosphorothioate deoxyCATGTC, linked to 2'-methoxy riboATTC, followed by p-ethoxy, 2'-methoxy riboTC linked to an inverted 3' terminal deoxythymidine); MMPT 89.12 (2'-methoxy riboCATGTCATTC linked to p-ethoxy, 2'-methoxy riboTC, linked to an inverted 3' terminal deoxythymidine); SST 89.12 (phosphorothioate deoxyCATGT linked to sulphur, 2'-methoxy riboCATTCTC linked to an inverted 3' terminal deoxythymidine); SOT 1.15 (phosphorothioate deoxyTGTGTA, linked to 2'-

methoxyriboGCCCATAGT, linked to an inverted 3' terminal deoxythymidine); SOT 5 (phosphorothioate deoxyTTGAC linked to 2'-methoxy riboATATCGGTCACTC linked to an inverted 3' terminal deoxythymidine); SOT 143.15 (phosphorothioate deoxyCTCATG linked to 2'-methoxyriboATTAACACC linked to an inverted 3' terminal deoxythymidine); SOM-89 (a sulphur, 2'-methoxyriboC, linked to phosphorothioate deoxyGCCA, linked to 2'-methoxyriboTGTCATTCTCCT, linked to sulphur, 2'-methoxyriboTAA, linked to a 3' terminal deoxyguanidine); SOM 72.1 (a 5' sulphur, 2'-methoxyriboA, linked to phosphorothioate deoxyCTGA, linked to 2'-methoxyriboTGACTTCATGAT, linked to sulphur, 2'-methoxyriboGCG, linked to a 3' terminal deoxycytosine); SOT 89.21 (phosphorothioate deoxyCGCCATGT linked to 2'-methoxyriboCATTCTCCTTAAG linked to an inverted 3' terminal deoxythymidine), SOM 114 (phosphorothioate deoxyGGAACGCG, linked to 2'-methoxyriboCCATTGGTA, linked to sulphur, 2'methoxyriboTAT, linked to a 3' terminal deoxycytidine), MMT 89.12 (2'-methoxyriboCATGTCATTCTC linked to an inverted 3' terminal deoxythymidine); 132 (SEQ ID NO. 15), SOM 1.1 (sulphur, 2'-methoxyriboA, linked to phosphorothioate deoxyGCAA, linked to 2'-methoxyriboCTGTGTAGCCCA, linked to sulphur, 2'-methoxyriboTAG, linked to a 3' terminal deoxythymidine, SOM 72.1, or SOM 5.1 (sulphur, 2'-methoxyT, linked to phosphorothioate deoxyACTT, linked to 2'-methoxyriboGACATATCGGTC, linked to sulphur, 2'-methoxyriboACT, linked to a 3' terminal deoxycytidine), and mixtures of SOT(5.15, 78.15, 89.15, and 114.15) or SOT(89.18, 114.15 (phosphorothioate deoxyCGCCAT linked to 2'-methoxyriboTGGTATATC linked to an inverted 3' terminal deoxythymidine), and 78.15 (phosphorothioate deoxyCATTGT linked to 2'-methoxyriboTTGTACTCC linked to an inverted 3' terminal deoxythymidine).

Figures 10a and 10b show the results of standard overnight MIC assays using the indicated oligonucleotides to test for antibacterial activity against *Staph. aureus*. Virtually all of the oligonucleotides tested (SOT-T12, SOT-

C12, SOT 89.(6, 9, and 12), SOC 89.12, SST 89.12, SOT 1.15, SOT 5.15 (phosphorothioate deoxyACATAT linked to 2'-methoxyriboCGGTCACTC linked to an inverted 3' terminal deoxythymidine), and SOT 143.15) significantly inhibited the growth of *Staph. aureus* (with the exception of the oligo dT string) relative to the control samples.

Figures 11a and 11b show the antibacterial activity of oligonucleotides DSM 89.18, SOT 78.15 (phosphorothioate deoxyCATTGT linked to 2'-methoxyriboTTGTACTCC linked to an inverted 3' terminal deoxythymidine), SOM 114.15, SOT 89.18 (phosphorthioate deoxyCATGTCAT linked to a 2'-methoxyriboTCTCCTTAAG, linked to an inverted 3' deoxythymidine), SOT 89.21, NBT 89.15, NBT 89.12-1 (phosphorothioate deoxyCATGTCATTCTC linked to a 3' terminal inverted phosphorothioate deoxythymidine), NMPT 89.12-2 (phosphorothioate deoxyCATGTCATTC linked to 2'-methyl, p-ethoxy TC, linked to an inverted 3' terminal deoxythymidine); MPT 89.12-4 (CATGTCATTCTC, with all p-ethoxy, 2'-methoxy RNA linked to an inverted 3' terminal deoxythymidine); MMPT 89.12-5 (2'-methoxy riboCATGTCATTC linked to p-ethoxy, 2'-methoxy riboTC, linked to an inverted 3' terminal deoxythymidine); SOT 89.12-6 (phosphorothioate deoxyCATGTC linked to 2'-methoxy riboATTCTC with an inverted 3' terminal deoxythymidine); SOPT 89.12-7 (phorphorothioate deoxyCATGTC linked to 2'-methoxy riboATTC followed by p-ethoxy, 2'-methoxy riboTC linked to an inverted 3' terminal deoxythymidine) when measured in standard overnight MIC assays against *Serratia liquefaciens*. As is readily apparent, all of the test oligonucleotides displayed significant antibacterial activity relative to controls.

Interestingly, the oligonucleotides used in Figures 10-11 retained antibacterial activity when used in standard overnight MIC assays over the three day time course. These data indicate that the tested antibacterial oligonucleotides are bactericidal for the test microorganisms.

Figure 12 shows the level of growth inhibition obtained when the oligonucleotides SOC 89.12, SOB 89.12, MMT 89.12,

MPT 89.12, SOPT 89.12, POT 89.12, DSM 89.18, SSM 89.18, NBT 89.15, NBPT 89.12, MMPT 89.12, SOT 89.12, and SOM-89F were tested in a standard MIC assay against *Staph. aureus*. All of the tested oligonucleotides proved effective at inhibiting the growth of *Staph. aureus*.

Figure 13 shows that several different length variants of SOT 89.21 (6, 12, 15, (phosphorothioate deoxyCATGTC linked to 2'-methoxy riboATTCTCCTT linked to an inverted 3' terminal deoxythymidine), and 18mers) were able to inhibit the growth of *Staph. aureus* when they were tested in a standard MIC assay against *Staph. aureus*.

Figures 14(a and b) compare the antibacterial activities of the conventional antibiotic ampicillin and SOT 114.21 against isolates of *Staph. aureus* strains 13301 and 29213.

Figure 15 shows that oligonucleotide MMT 114.15 (2'-methoxyriboCGCCATTGGTATATC linked to an inverted 3' terminal deoxythymidine) proved capable of inhibiting the growth of *P. aeroginosa* strain 10145, an opportunistic Gram negative pathogen that has proved resistant to many conventional antibiotics, in a standard MIC assay.

Figure 16 shows that oligonucleotide SOT 114.21 proved capable of inhibiting the growth of the pathogen *Strep. pyogenes* strain 14289 in a standard MIC assay.

EQUIVALENTS

The foregoing specification is considered to be sufficient to enable one skilled in the art to broadly practice the invention. Indeed, various modifications of the above-described methods for carrying out the invention which are obvious to those skilled in the field of microbiology, biochemistry, organic chemistry, medicine or related fields are intended to be within the scope of the following claims. All patents, patent applications, and publications cited are incorporated herein by reference.

TABLE 1

Table 1

Antimicrobial Susceptibility According to NCCLS Standards

Gene/ Operon Target	NBT Number	Drug R Gram Neg	Drug R Gram Pos	SEQ ID NO.	Sequence
Category of Target: Energy Metabolism					
hemA	NBT 28	97% INH	100% INH	1	AAG GGT CAT GTC TGC GGG AAA TAA TAC
aroC	NBT 32	97% INH	57% INH	2	CCG TTA TTG TTG TGT TTG CGT GTT TAC
aroA	NBT 36			3	CAG GGA TTC CAT GAA ACT CAA CTC TCA
chaC	NBT 47			4	ACA CTT CCG CCA CTG CAT ACT TCC CTG
chaB	NBT 48			5	TCG TTT TAT ACG GCA TCG TTG ACT CCT
chaA	NBT 49			6	GAC ATT ATG GTT ATC CCT TTG CAG ATG
ATP operon	NBT 57	56% INH		7	TTC ACT CCT GCT CCC TTC GAG GTA TGC
hemD	NBT 61			8	GCG GGT GAC AAG GAT ACT CAT GCC GGG
hemX	NBT 62			9	CAT TAT GGC TTC CTG TTA TGA GAG TTA
moa operon	NBT 67			10	GTT GTG AAG CCA TGT ACA CCT TTC CAG
crp	NBT 84	78% INH	26% INH	11	GTT TGC CAA GCA CCA TGC GCG GTT TAC
ATPase	NBT 88	72% INH		12	CGT CAT ATT TTC TGA AGC CAT GAT GCC
cya	NBT 104			13	GGT ACA AGA CGT ATC GCC TGA TTT GCT
pckA	NBT 126			14	CAT TTC TCA GCT CCT TAG CCA ATA TGT
fadD	NBT 132	89% INH	100% INH	15	AGC CAA ACC TTC TTC AAT TCT TCA CCT
Category of Target: DNA Replication					
gyrA	NBT 9 NBT 10	100% INH	33% INH	16 17	AAG GTC GCT CAT CTA ACC GCT ATC CCT AGG TAA TTC AGC CAT CAA GAG TTC CTC
gyrB	NBT 11	96% INH	100% INH	18	AAT GCA GTC ACC ATC GCT TTC TGT TAC
lig	NBT 26			19	GCA TCA GCC TGT CGT ATT CAG CGT CGG
dnaG	NBT 30			20	CGG CTC GTT TTC ACG TAC TTT AAT TAC
ssb	NBT 37			21	TCT GCT GGC CAT AAT TGA GTC TCC TGA
groESL	NBT 66	63% INH		22	ATA ACT CTC CTT TGA GAA AGT CCG TAT
dna A operon	NBT 79	65% INH		23	AAG CGA AAG TGA CAC GGC GGA CTC CAC
dnaT operon	NBT 81			24	GGT CAT CAA GAT CAT TCG GGA ACC ATG
parC	NBT 95			25	TCG CTC ATT AAT TCT GAT TCC TCA ACT
holD	NBT 109			26	TAA CTG CCA GTC TCG TCG GGA TGT CAT
dnaQ	NBT 124			27	CGT GTA ATT GCA GTG CTC ATA GCG GTC
dnaE	NBT 130			28	TGT ACG AAA CGT GGT TCA GAC ATC TTC
dnaJ	NBT 133			29	CTC GTA ATA ATC TTG CTT AGC CAT CTT
Category of Target: Cell Division Control					
minB	NBT 39	100% INH	34% INH	30	TGA CAT CCT GGC CTT ACT CAA TTA GCT
minD	NBT 40			31	CAA CAA TAA TGC GTG CCA TAG AAA TTC
minE	NBT 41			32	GAG TAA TGC CAT AAC TTA TCC TCC GAA
ftsW	NBT 42			33	AAC GCA TCA ACC TAA CTC CTT CGC CAG
ftsN	NBT 43	100% INH		34	TAT TTA TTC GTT CGT CAG CCC GCC ATG

Table 1

		Antimicrobial Susceptibility According to NCCLS Standards			
ftsH	NBT 44			35	GAA CGC TTC TTA CCT GTC ATT TAA CTT
ftsJ	NBT 45			36	GTA TTA GGT TTT TCG CCA TGT CAC TCA
ftsQ	NBT 52			37	TTC AGA GCA GCC TGC GAC ATA TTA GAC
ftsA	NBT 53			38	CGT CGC CTT GAT CAT TGT TGT TCT GCC
ftsZ	NBT 54			39	ATT GGT TCA AAC ATA GTT TCT CTC CGA
parB	NBT 55			40	TGG ATG TTT CAT GGC CTT CTC CTT
fts YEX operon	NBT 65	60% INH	21% INH	41	CTA CAC TCC TCG CTG TTG CTT CAT GGC
pbpB	NBT 80	90% INH	42% INH	42	TGC TTT CAT GCG TCG CGT TTA TCC TTA
rodA	NBT 83			43	TTA TCC GTC ATG ATT AAT GGT CCT CCG
tig	NBT 119			44	GCA TCT TGT TAC CTC AAA AAA TCA CAG
Category of Target: Regulatory Proteins					
lon	NBT 27	97% INH	100% INH	45	AGG ATT CAT AGA GCT CTC TAG TTT
rel B	NBT 56			46	TTA ACA TCT TTT GCT GCT GCT TCA TAG
crp	NBT 84	78% INH	26% INH	47	GTT TGC CAA GCA CCA TGC GCG GTT TAC
lexA	NBT 131			48	GCC TGG CCG TTA ACG CTT TCA TTC CGC
Category of Target: Cell Wall Biosynthesis					
asd	NBT 1 NBT 2 NBT 3 NBT 7 NBT 8	97% INH	100% INH	49 50 51 52 53	AAC GAT AGC AAC TGT GTA GCC CAT AGT ACG AGT TCC ACC GGC GCC TGT AGC ACC GGA GCC GAC CAT ACC GCG CCA GCC GAT CAA CAA CTG CGA TGG TGG TCA TTG TAA ACC GCG CCA GCC GAC GAA ACC TAC TTT
ddlA	NBT 33 NBT 34 NBT 35	70% INH	26% INH	54 55 56	CGT CTA ACA CAA AGT GCA TAC ATT ACC CAC TTC ATG TTG CGC TGA TTT ACC ACC CCA TCT TAA AAA CCT ATC CCG TCT AAC
murG	NBT 50			57	CTT GAC CAC TCA TCG TGA ACC TCG TAC
murC	NBT 51			58	GTT GTG TAT TCA TTC TTT ACG CCA TTA
lysA	NBT 6			59	CAG TGA ATG TGG CAT AAC AAA CTC CAG
murD	NBT 139			60	CGT ACC TTC AGC GTT GCC AGA CCA ATC
Category of Target: Sugar Metabolism					
zwf	NBT 89	69% INH	100% INH	61	CGT TAC CGC CAT GTC ATT CTC CTT AAG
sdhB	NBT 112			62	CTG TCT CCG CAT TAG TAA GTA CGA ATC
Category of Target: Virulence, Pili, Flagella					
pap operon	NBT 72	31% INH		63	TGA CCG ACT GAT GAC TTC ATG ATG CGC
rim J	NBT 103	76% INH		64	CAT TCT ATA CCT ACT CCT TCC CGT AAC
Category of Target: Fatty Acid Metabolism					
fadD	NBT 132	89% INH	100% INH	65	AGC CAA ACC TTC TTC AAT TCT TCA CCT
Category of Target: mRNA Synthesis/Stability					
rpoN operon	NBT 19	100% INH	23% INH	66	TAG GAT GTT CTA ACC TTT TCA ATC AGC
alpha operon	NBT 29	98% INH		67	TAC GGG CCA CTA TGC ACT CCT ACT ATT
MMS operon	NBT 30			68	CGG CTC GTT TTC ACG TAC TTT AAT TAC
rho	NBT 125			69	GAT TCA TAG TGG TGT GAG TTC TTA AAC
rnpB	NBT 121			70	GAA GAG GAC GAC GAC GAA GCG GCG ACT

Table 1

		Antimicrobial Susceptibility According to NCCLS Standards			
ams	NBT 134			71	TCA TCG TAA CTT ACT CAT TAT TCT TAC
Category of Target: tRNA Synthesis					
trm D	NBT 16	100% INH	100% INH	72	GCT AAA CGA ATA GTT ACC ATA ACA TCC
met Y	NBT 18 18.12 18.15 18.18	100% INH	100% INH	73 174 175 176	TAA TCA TCT CTG CTA ATT TTG CTC TAA TCA TCT CTG CTA TCA TCT CTG CTA ATT TCA TCT CTG CTA ATT TTG
val U operon	NBT 91 NBT 92			74 75	GTG CTC TCC CAG CTG AGC TAA TCA CCC CTC TCC CAG CTG AGC TAA TCA CCC
tRNA operon	NBT 93 NBT 94			76 77	CGC TCT ATC CAG CTG AGC TAC GGG CGC TCT ATC CAG CTG AGC TAC GGG CGC
infA operon	NBT 100			78	TCA CAA TAA ACT CCT TAC CAT CCC ATT
Category of Target: rRNA Synthesis					
rrnB operon	NBT 96	80% INH		79	GCC GCC AGC GTT CAA TCT GAG TGA
Category of Target: Ribosomal Protein Synthesis					
str operon	NBT 12	97% INH		80	AAC TGT TGC CAT TAA ATA GCT CCT GGA
slO operon	NBT 15	100% INH	40% INH	81	GCG GAT ACG GAT TCT TTG GTT CTG CAT
trmD operon	NBT 16	100% INH	100% INH	82	GCT AAA CGA ATA GTT ACC ATA ACA TCC
spc operon	NBT 17	100% INH	98% INH	83	GTT CAG CAT AGT CTG TTC TTG GAT CAT
S15 operon	NBT 20	99% INH	100% INH	84	AGC TGT TGC TTC AGT ACT TAG AGA CAT
S12 operon	NBT 21	82% INH	100% INH	85	TTG TAG GCA TCT ACA TTC TCC TGT GTT
alpha operon	NBT 29	98% INH		86	TAC GGG CCA CTA TGC ACT CCT ACT ATT
MMS operon	NBT 30			87	CGG CTC GTT TTC ACG TAC TTT AAT TAC
tsf	NBT 38	67% INH	46% INH	88	AAA CAG TTG CCA TGA TTA TTT CCT CTA
rim J	NBT 103	78% INH		89	CAT TCT ATA CCT ACT CCT TCC CGT AAC
rim I	NBT 107 NBT 108			90 91	TTT CGA GGG AAG AAA TCG TGT TCA TAT GTG GTA AGC CGC CGG TAA ATC AGT CGT
rnpA	NBT 122			92	TTA CTT AGA AAC GGT CAG ACG AGC GCG
rpmH	NBT 123			93	GCG TTT CAT GGC GAT TTC TAC CTA AAC
Category of Target: Protein Synthesis					
str operon	NBT 12	97% INH		94	AAC TGT TGC CAT TAA ATA GCT CCT GGA
nusA operon	NBT 18	100% INH	100% INH	95	TAA TCA TCT CTG CTA ATT TTG CTC TAA
hemA	NBT 28	97% INH	100% INH	96	AAG GGT CAT GTC TGC GGG AAA TAA TAC
infC	NBT 31			97	TCC GCC TTT AAT ACC TTA TTC CTC CAA
tsf	NBT 38	100% INH	46% INH	98	AAA CAG TTG CCA TGA TTA TTT CCT CTA
prf	NBT 90			99	GGC TTC ATA GGC GTA AAT TCA CCC TGT
infA operon	NBT 110			100	GCA ACA AAC AGG TTC GGC ACA TTA CTC

Table 1

		Antimicrobial Susceptibility According to NCCLS Standards			
aat	NBT 135			101	TAT TGA ATG GCG AGA AAG CAG AAC CAG
Category of Target: Phospholipid Synthesis					
adk	NBT 25	96% INH	44% INH	102	CCG AAG CAG AAT AAT ACG CAT TAC GAA
psd	NBT 105	83% INH	50% INH	103	AAA TGA ATT TAA CAA GGT AGC CTC CAG
pss	NBT 106			104	CAG TGC ATT TCT TCT CTG TTC ATT GAA
Category of Target: Periplasmic/Secretory Proteins					
envA	NBT 46	59% INH	25% INH	105	TTT GTT TGA TCA TCG TAT TAT CTC GCC
tolA	NBT 85			106	CGG TTG CCT TTG ACA CTC TCG GTT TCC
tolB	NBT 86			107	CCT GCT TCA TCA TAT CTC CCT ATA CTG
secA	NBT 118			108	CTT TAG TTA ACA ATT TGA TTA GCA TAA
Category of Target: Transport Proteins					
biotin operon	NBT 58 NBT 59 NBT 60	84% INH		109 110 111	GCG ACA ATG TCC AGC GTG GGC GGT GAG ATC GGG CTT CTC CAA AAT ATG TTG TTT GTT AAT TCG GTG TAG ACT TGT AAA CCT
fhuA	NBT 114	100% INH	18% INH	112	GGA ACG CGC CAT TGG TAT ATC TCT GAT
fhuC	NBT 115			113	TCC TGC ATA ACA GCC AAC TTG TGA TTA
jhuD	NBT 116			114	TAA GAG GTA AGC CGC TCA TCA ATA AAC
fhuB	NBT 117			115	CTG CGA GAA GTT CAT CCA GGT GAG CGC
Category of Target: Amino Acid Biosynthesis					
aroC	NBT 32	97% INH		116	CCG TTA TTG TTG TGT TTG CGT GTT TAC
aroA	NBT 36			117	CAG GGA TTC CAT GAA ACT CAA CTC TCA
nir operon	NBT 71	93% INH	42% INH	118	ATA ATT GCG AGT CTG ACT TTG CTC ATT
asd	NBT 1 NBT 2 NBT 3 NBT 7 NBT 8	97% INH	100% INH	119 120 121 122 123	AAC GAT AGC AAC TGT GTA GCC CAT AGT ACG AGT TCC ACC GGC GCC TGT AGC ACC GGA GCC GAC CAT ACC GCG CCA GCC GAT CAA CAA CTG CGA TGG TGG TCA TTG TAA ACC GCG CCA GCC GAC GAA ACC TAC TTT
Category of Target: Lipopolysaccharide Synthesis					
rfaY	NBT 73	100% INH	36% INH	124	GTC TTT GAT CTT GCT CTT CTG AAT CAT
rfaZ	NBT 74			125	TAT CTA ATA TTC TTC ATG ATA AAC CTG
rfaL	NBT 75			126	TTC CTA AGC GCA TTT TTA TAC CAT ATT
rfaK	NBT 76			127	TAA TGA TGA TAA CTT TTC CAA AAC TGC
l ps operon	NBT 77	76% INH		128	CCA TGA TAT CGC ATC TTT ATG ACC AGG
Category of Target: Purine/Pyrimidine Biosynthesis					
adk	NBT 25	96% INH		129	CCG AAG CAG AAT AAT ACG CAT TAC GAA
deoC operon	NBT 63	100% INH	51% INH	130	GCT TTC AGA TCA GTC ATT TCA TTC TCC
pyrE operon	NBT 64			131	TTC ATC ATA ACG GGT CAC GAT CTC GTC
prs	NBT 111	87% INH		132	CAT ATC AGG CAC CAG AAG AAC CTC AGG
gpt	NBT 128			133	TTC GCT CAT GTG AAG TGT CCC AGC CTG
Category of Target: Outer Membrane Proteins					
ompB operon	NBT 78	100% INH	33% INH	134	GTA GTT CTC TTG CAT TGT TTG TAC TCC
nlpA	NBT 87			135	GGC TAG ATG ATG TGT TGT CAG TTT CAT

Table 1

		Antimicrobial Susceptibility According to NCCLS Standards			
ompX	NBT 97			136	CAT AAC CAC CTC AAA TGT GAT TCA AAT
ompF	NBT 98			137	GCC AGA ATA TTG CGC TTC ATC ATT ATT
ompC	NBT 99			138	TAA CTT TCA TGT TAT TAA CCC TCT GTT
ompH	NBT 100			139	TCA CAA TAA ACT CCT TAC CAT CCC ATT
ompP	NBT 101			140	CAG AAG CTT AGT TTG CAT AAC AAT GAC
ompA	NBT 102			141	AAT CGC GAT AGC TGT CTT TGT CAT
tsx	NBT 120			142	CAT ATG TAT GCC ACT GTT TGA AAA TCC
lpp	NBT 127	91% INH	98% INH	143	GCC CAG TAC CAG TTT AGT AGC TTT CAT
envM	NBT 129			144	ACC CAT AGC TTT AAT CCT TAT TGT TGA
envC	NBT 137			145	GCA TGT TTC GTC ATT ACT ATT CCT CAA
envD	NBT 138			146	GTT TGC CAT GTC AGA TTA CCT TAC TTC
envR	NBT 136			147	TTT GCC ATG ATT AAT TAT TCA GGA AAT
Category of Target: Nitrate Reductase					
nar operon	NBT 68	70% INH		148	ATT TAC TCA TCG GTT TTC TCC TGT GGG
nar XL operon	NBT 69			149	AAG CAT GTA AAC CTC TTC CTT CAG GCT
nar ZYWZ operon	NBT 70			150	GAT CCA AAA GTT TAC TCA TAG CAT GAC
nir operon	NBT 71	80% INH	42% INH	151	ATA ATT GCG AGT CTG ACT TTG CTC ATT
Category of Target: Drug Resistance					
sulA	NBT 5	100% INH	100% INH	152	TGG CTT TAC TTG ACA TAT CGG TCA CTC
str operon	NBT 12			153	AAC TGT TGC CAT TAA ATA GCT CCT GGA
bla	NBT 14	99% INH	98% INH	154	ACA CGG AAA TGT TGA ATA CTC ATA CTC
spc operon	NBT 17	100% INH	98% INH	155	GTT CAG CAT AGT CTG TTC TTG GAT CAT
S12 operon	NBT 21	82% INH	100% INH	156	TTG TAG GCA TCT ACA TTC TCC TGT GTT
tet resistance	NBT 22	100% INH	90% INH	157	ATT GTT AGA TTT CAT ACA CGG TGC CTG
kan resistance	NBT 23	98% INH	10% INH	158	CAT CTT GTT CAA TCA TGC GAA ACG ATC
ermC	NBT 24			159	ACT GTG TTT TAT ATT TTT CTC GTT CAT
pbpB	NBT 80	90% INH	42% INH	160	TGC TTT CAT GCG TCG CGT TTA TCC TTA
pbpA	NBT 82			161	AAG AGT TCT GTA GTT TCA TCC GCT GCG
Category of Target: Vitamin Metabolism					
biotin operon	NBT 58 NBT 59 NBT 60	84% INH		162 163 164	GCG ACA ATG TCC AGC GTG GGC GGT GAG ATC GGG CTT CTC CAA AAT ATG TTG TTT GTT AAT TCG GTG TAG ACT TGT AAA CCT
folic acid	NBT 5	100% INH	100% INH	165	TGG CTT TAC TTG ACA TAT CGG TCA CTC
Category of Target: Miscellaneous					
IS	NBT 144	100% INH		166	CCT CAT CAA ACA ATG

Table 1

		Antimicrobial Susceptibility According to NCCLS Standards			
$(AT)_n$	NBT 140	100% INH		167	ATA TAT ATA TAT ATA TAT
$(AC)_n$	NBT 141	100% INH		168	ACA CAC ACA CAC ACA CAC
$(TC)_n$	NBT 142	100% INH		169	TCT CTC TCT CTC TCT CTC
$(T)_{11}$	NBT 13	100% INH		170	TTT TTT TTT TTT TTT TTT
$(C)_{11}$	NBT 143	100% INH		171	CCC CCC CCC CCC CCC CCC
sucA	NBT 113			172	CAA AGC GCT GTT CTG CAT CGT GAT CCC
(RS)	NBT 4			173	GAT ATC CGC ATG GTT CAA CAG ATG ACA

Table 2A. Energy Metabolism - Oligonucleotide #28

Escherichia coli 35218 Multiple Drug Resistance			
Time *T=0*	Control 0	28 0.001	%Inhib
0	0	0	
60	0.002	0.001	50%
120	0.001	0.001	0%
180	0.003	0	100%
240	0.008	0	100%
285	0.015	0	100%
320	0.026	0	100%
350	0.04	0	100%
380	0.058	0.001	98%
410	0.076	0.002	97%
430	0.091	0.004	96%
450	0.105	0.004	96%

Table 2B. DNA Replication - Oligonucleotide #10

Escherichia coli 35218 Multiple Drug Resistance			
Time *T=0*	Control 0	10 0.003	%Inhib
0	0	0	
60	0.001	0.001	0%
120	0	0	
170	0.008	0	100%
230	0.008	0	100%
170	0.017	0	100%
305	0.025	0	100%
340	0.046	0	100%
365	0.058	0	100%
385	0.075	-0.002	103%
400	0.082	-0.002	102%
415	0.094	-0.002	102%
425	0.105	0.001	99%

Table 2C. Cell Division Control - Oligonucleotide #43

Escherichia coli 35218 Multiple Drug Resistance			
Time T=0	Control 0	43 0.005	%Inhib
0	0	0	
105	0.002	-0.001	150%
175	0.003	-0.004	233%
220	0.004	-0.003	175%
270	0.007	-0.003	143%
300	0.012	-0.003	125%
330	0.022	-0.003	114%
360	0.032	-0.002	106%
395	0.052	-0.001	102%
425	0.065	0	100%
445	0.081	0.001	99%
465	0.09	0.002	98%
490	0.108	0.008	93%

Table 2D. Regulatory Proteins - Oligonucleotide #27

Escherichia coli 35218 Multiple Drug Resistance			
Time T=0	Control 0	27 0.002	%Inhib
0	0	0	
60	0.002	0.001	50%
120	0.001	0.001	0%
180	0.003	0	100%
240	0.008	0	100%
285	0.015	0	100%
320	0.026	0	100%
350	0.04	-0.001	103%
380	0.058	0.001	98%
410	0.076	0.002	97%
430	0.091	0.003	97%
450	0.105	0.003	97%

Table 2E. Cell Wall Biosynthesis - Oligonucleotide #2

Escherichia coli 35218 Multiple Drug Resistance			
Time T=0	Control 0	2 0.002	% Inhib
0	0	0	
105	0.002	-0.001	150%
175	0.003	-0.002	167%
220	0.004	-0.001	125%
270	0.007	-0.001	114%
300	0.012	-0.001	108%
330	0.022	-0.001	105%
360	0.032	0	100%
395	0.052	0	100%
425	0.065	0	100%
445	0.081	0.002	98%
465	0.09	0.003	97%
490	0.108	0.008	93%

Table 2F. Sugar Metabolism - Oligonucleotide #89

Staphylococcus aureus 13301			
Time T=0	Control 0	89 0.003	% Inhib
0	0	0	
90	0.002	-0.002	200%
150	0.004	-0.002	150%
210	0.008	-0.002	125%
255	0.015	-0.002	113%
285	0.026	-0.001	104%
315	0.039	-0.001	103%
345	0.052	-0.001	102%
375	0.073	-0.002	103%
395	0.08	-0.001	101%
415	0.089	-0.002	102%
435	0.103	-0.002	102%

Table 2G. Virulence, Pili, Flagella - Oligonucleotide #103

Escherichia coli 35218 Multiple Drug Resistance			
Time *T=0*	Control *0*	103 *0.004*	% Inhib
0	0	0	
60	0.001	-0.001	200%
120	0.002	-0.002	200%
180	0.006	-0.001	117%
215	0.012	-0.001	108%
250	0.02	0	100%
285	0.031	0.001	97%
325	0.072	0.009	88%
355	0.085	0.015	82%
375	0.096	0.021	78%
395	0.108	0.026	76%

Table 2H. Fatty Acid Metabolism - Oligonucleotide #132

Escherichia coli 35218 Multiple Drug Resistance			
Time *T=0*	Control *0*	132 *0.003*	% Inhib
0	0	0	
60	0.001	-0.003	400%
120	0.004	-0.002	150%
165	0.007	-0.003	143%
205	0.018	-0.002	111%
235	0.028	-0.002	107%
265	0.039	-0.001	103%
295	0.063	0.003	95%
315	0.078	0.004	95%
335	0.093	0.009	90%
355	0.107	0.013	88%

Table 2I. mRNA Synthesis/Stability - Oligonucleotide #19

Escherichia coli 35218 Multiple Drug Resistance			
Time *T=0*	Control -0.001	19 0.005	% Inhib
0	0	0	
60	0.001	-0.001	200%
150	0.002	-0.001	150%
195	0.005	-0.001	120%
245	0.013	-0.002	115%
275	0.019	-0.001	105%
320	0.04	0	100%
350	0.054	-0.002	104%
365	0.066	0	100%
385	0.079	-0.002	103%
415	0.095	0.003	97%
430	0.105	0.001	99%

Table 2J. tRNA Synthesis - Oligonucleotide #16

Escherichia coli 35218 Multiple Drug Resistance			
Time *T=0*	Control 0	16 0.003	% Inhib
0	0	0	
60	0.001	-0.002	300%
120	0	-0.002	
170	0.003	-0.002	167%
170	0.008	-0.002	125%
275	0.017	-0.004	187%
305	0.025	-0.004	185%
305	0.025	-0.004	109%
365	0.058	-0.004	107%
385	0.075	-0.004	105%
400	0.082	-0.004	105%
415	0.094	-0.004	104%
425	0.105	-0.002	102%

Table 2K. rRNA Synthesis - Oligonucleotide #96

Escherichia coli 35218 Multiple Drug Resistance			
Time T=0	Control 0	96 0.005	% Inhib
0	0	0	
60	0.002	-0.002	200%
120	0.004	-0.005	225%
165	0.005	-0.004	180%
210	0.011	-0.003	127%
250	0.018	-0.002	111%
275	0.025	-0.001	104%
305	0.037	0.003	92%
340	0.056	0.013	77%
360	0.069	0.02	71%
380	0.08	0.028	65%
400	0.096	0.042	56%
420	0.108	0.053	51%

Table 2L. Ribosomal Protein Synthesis - Oligonucleotide #21

Escherichia coli 35218 Multiple Drug Resistance			
Time T=0	Control 0	21 0.002	% Inhib
0	0	0	
60	0.001	-0.003	400%
120	0.004	-0.002	150%
165	0.007	-0.004	157%
205	0.018	-0.001	106%
235	0.028	-0.001	104%
265	0.039	0.001	97%
295	0.063	0.007	89%
315	0.078	0.01	87%
335	0.093	0.018	81%
355	0.107	0.025	77%

Table 2M. Protein Synthesis - Oligonucleotide #18

Escherichia coli 35218 Multiple Drug Resistance			
Time *T=0*	Control 0.001	18 0.017	% Inhib
0	0	0	
60	0.001	-0.004	500%
120	0.002	-0.004	300%
165	0.005	-0.009	280%
210	0.015	-0.01	167%
255	0.025	-0.012	148%
285	0.041	-0.01	124%
315	0.058	-0.011	119%
335	0.073	-0.009	112%
355	0.089	-0.007	108%
375	0.101	-0.006	106%

Table 2N. Phospholipid Synthesis - Oligonucleotide #105

Escherichia coli 35218 Multiple Drug Resistance			
Time *T=0*	Control 0	105 0.003	% Inhib
0	0	0	
60	0.001	-0.003	400%
120	0.003	-0.003	200%
180	0.008	-0.002	125%
225	0.015	-0.003	120%
260	0.026	0	100%
285	0.033	0.002	94%
315	0.047	0.008	83%
335	0.062	0.012	81%
355	0.075	0.022	71%
375	0.085	0.026	69%
395	0.101	0.04	60%

Table 2O. Periplasmic/Secretory Proteins - Oligonucleotide #46

Escherichia coli 35218 Multiple Drug Resistance			
Time T=0	Control 0.002	46 0.004	% Inhib
0	0	0	
60	0.001	0.001	0%
120	0.001	0.002	-100%
180	0.001	0	100%
240	0.005	0.001	80%
285	0.012	0.001	92%
350	0.027	0.003	89%
390	0.043	0.012	72%
420	0.063	0.018	71%
450	0.082	0.028	66%
470	0.096	0.039	59%
500	0.106	0.046	57%

Table 2P. Transport Proteins - Oligonucleotide #114

Salmonella typhimurium 23564			
Time T=0	Control 0.004	114 0.008	% Inhib
0	0	0	
60	-0.001	0	
120	0	-0.002	
165	0	-0.004	
230	0.003	-0.004	233%
260	0.005	-0.004	180%
305	0.014	-0.002	114%
335	0.021	0	100%
365	0.033	0.001	97%
395	0.052	0.007	87%
415	0.066	0.012	82%
435	0.08	0.018	78%
455	0.093	0.026	72%
476	0.108	0.035	68%

Table 2Q. Amino Acid Biosynthesis - Oligonucleotide #32

Escherichia coli 35218 Multiple Drug Resistance			
Time T=0	Control 0	32 0.002	% Inhib
0	0	0	
60	0.002	0.001	50%
120	0.001	0	100%
180	0.003	0.001	67%
240	0.008	0.001	88%
285	0.015	0	100%
320	0.026	0	100%
350	0.04	0	100%
380	0.058	0.002	97%
410	0.076	0.002	97%
430	0.091	0.003	97%
450	0.105	0.003	97%

Table 2R. Lipopolysaccharide Synthesis - Oligonucleotide #73

Escherichia coli 35218 Multiple Drug Resistance			
Time T=0	Control 0.006	73 0.005	% Inhib
0	0	0	
60	0	0	
120	0.001	0	100%
165	0.001	0	100%
210	0.005	-0.001	120%
240	0.008	0	100%
275	0.015	0	100%
305	0.024	-0.001	104%
335	0.034	0	100%
365	0.048	0.001	98%
390	0.061	0.003	95%
410	0.07	0.003	96%
430	0.086	0.005	94%
455	0.1	0.01	90%

Table 2S. Purine/Pyrimidine Biosynthesis - Oligonucleotide #63

Escherichia coli 35218 Multiple Drug Resistance			
Time T=0	Control 0.002	63 0.004	% Inhib
0	0	0	
60	0.001	0.001	0%
120	0.001	0.002	-100%
180	0.001	0.001	0%
240	0.005	0.002	60%
285	0.012	0.001	92%
350	0.027	-0.001	104%
390	0.043	0.001	98%
420	0.063	0.002	97%
450	0.082	0.001	99%
470	0.096	0.004	96%
500	0.106	0.008	92%

Table 2T. Outer Membrane Proteins - Oligonucleotide #78

Escherichia coli 35218 Multiple Drug Resistance			
Time T=0	Control 0.001	78 0.004	% Inhib
0	0	0	
60	0.001	-0.002	300%
120	0.002	-0.002	200%
165	0.005	-0.003	160%
210	0.015	-0.004	127%
255	0.025	-0.004	116%
285	0.041	-0.003	107%
315	0.058	-0.003	105%
335	0.073	-0.002	103%
355	0.089	-0.002	102%
375	0.101	-0.002	102%

Table 2U. Nitrate Reductase - Oligonucleotide #71

Escherichia coli 35218 Multiple Drug Resistance			
Time T=0	Control 0	71 0.002	% Inhib
0	0	0	
105	0.002	0	100%
175	0.003	-0.002	167%
220	0.004	-0.001	125%
270	0.007	-0.001	114%
300	0.012	-0.001	108%
330	0.022	-0.001	105%
360	0.032	0	100%
395	0.052	0	100%
425	0.065	0.003	95%
445	0.081	0.004	95%
465	0.09	0.006	93%
490	0.108	0.013	88%

Table 2V. Drug Resistance - Oligonucleotide #114

Escherichia coli 35218 Multiple Drug Resistance			
Time T=0	Control 0	114 0.006	% Inhib
0	0	0	
105	0.002	-0.002	200%
175	0.003	-0.005	267%
220	0.004	-0.003	175%
270	0.007	-0.003	143%
300	0.012	-0.004	133%
330	0.022	-0.004	118%
360	0.032	-0.004	113%
395	0.052	-0.004	108%
425	0.065	-0.003	105%
445	0.081	-0.001	101%
465	0.09	0	100%
490	0.108	0.004	96%

Table 2W. Vitamin Metabolism - Oligonucleotide #5

Escherichia coli 35218 Multiple Drug Resistance			
Time *T=0*	Control -0.001	5 0.002	% Inhib
0	0	0	
60	0.001	-0.001	200%
150	0.002	-0.003	250%
195	0.005	-0.002	140%
245	0.013	-0.001	108%
275	0.019	0	100%
320	0.04	0	100%
350	0.054	-0.001	102%
365	0.066	0	100%
385	0.079	0	100%
415	0.095	-0.001	101%
430	0.105	0.001	99%

Table 3A.

Escherichia coli 35218 Multiple Drug Resistance							
Time *T=0*	Control 0	A 0.003	%Inhib	B 0.006	%Inhib	C 0.003	%Inhib
0	0	0		0		0	
60	0.001	-0.001	200%	-0.004	500%	-0.002	300%
105	0.002	-0.002	200%	-0.004	300%	-0.002	200%
145	0.002	-0.001	150%	-0.003	250%	-0.002	200%
190	0.002	-0.001	150%	-0.003	250%	-0.002	200%
230	0.005	-0.001	120%	-0.003	160%	-0.002	140%
275	0.009	-0.001	111%	-0.003	133%	-0.003	133%
320	0.015	-0.002	113%	-0.002	113%	-0.003	120%
350	0.022	-0.001	105%	-0.001	105%	-0.003	114%
380	0.03	0	100%	-0.001	103%	-0.002	107%
410	0.048	0.001	98%	-0.001	102%	-0.003	106%
445	0.068	0.005	93%	-0.003	104%	-0.003	104%
465	0.08	0.009	89%	-0.002	103%	-0.003	104%
485	0.097	0.015	85%	0.002	98%	-0.003	103%

A=2'-O-Me version 18

B=12mer version 18

C=15mer version 18

Table 3B.

Escherichia coli 35218 Multiple Drug Resistance					
Time T=0	Control 0	D 0.002	%Inhib	E 0.006	%Inhib
0	0	0		0	
60	0.001	-0.001	105%	-0.004	500%
105	0.002	-0.001	150%	-0.004	300%
145	0.002	-0.001	150%	-0.005	350%
190	0.002	-0.001	107%	-0.002	200%
230	0.005	-0.001	120%	-0.004	180%
275	0.009	-0.001	111%	-0.004	144%
320	0.015	-0.001	107%	-0.004	127%
350	0.022	-0.001	105%	-0.003	114%
380	0.03	-0.001	103%	-0.003	110%
410	0.048	-0.001	102%	-0.003	106%
445	0.068	-0.001	101%	-0.001	101%
465	0.08	-0.001	101%	-0.001	101%
485	0.097	-0.001	101%	0.002	98%

D=5'amino group/15mer version 18

E=33mer version 18

Table 3C.

Staphylococcus aureus 13301							
Time T=0	Control 0	A 0	%Inhib	B -0.001	%Inhib	C 0.001	%Inhib
0	0	0		0		0	
90	0.003	0.002	33%	0.003	0%	0.003	0%
150	0.003	0.001	67%	0.004	-33%	0.003	0%
210	0.005	0.002	60%	0.004	20%	0.003	40%
270	0.006	0.001	83%	0.003	50%	0.003	50%
325	0.014	0.001	93%	0.002	86%	0.003	79%
380	0.032	0.002	94%	0.003	91%	0.002	94%
410	0.044	0.003	93%	0.003	93%	0.003	93%
440	0.057	0.004	93%	0.003	95%	0.003	95%
470	0.075	0.005	93%	0.021	72%	0.003	96%
500	0.105	0.011	90%	0.004	96%	0.004	96%

A=2'-O-Me version 18

B=pEthoxy version 18

C=12mer version 18

Table 3D.

Staphylococcus aureus 13301					
Time *T=0*	Control 0.001	D 0.003	%Inhib	E 0.003	%Inhib
0	0	0		0	
65	0.001	0.001	0%	0.003	-200%
125	0.002	0.003	-50%	0.003	-50%
185	0.003	0.002	33%	0.004	-33%
240	0.003	0.002	33%	0.004	-33%
295	0.004	0.002	50%	0.003	25%
340	0.007	0.003	57%	0.006	14%
385	0.011	0.003	73%	0.005	55%
415	0.016	0.002	88%	0.004	75%
445	0.021	0.002	90%	0.004	81%
475	0.032	0.002	94%	0.004	88%
505	0.029	0.002	93%	0.005	83%
535	0.045	0.002	96%	0.006	87%
565	0.057	0.002	96%	0.005	91%
595	0.072	0.002	97%	0.009	88%
625	0.09	0.002	98%	0.006	93%

D=15mer version 18

E=18mer version 18

Table 4A.

Salmonella typhimurium												
Time T=0	Control -0.001	18 0.004	%Inhib	39 0.004	%Inhib	63 0.003	%Inhib	78 0.002	%Inhib
0	0	0		0		0		0	
90	0.001	-0.001	200%	-0.002	300%	-0.002	300%	-0.001	200%
150	0.002	-0.004	300%	-0.002	200%	-0.001	150%	-0.001	150%
210	0.003	-0.004	233%	-0.002	167%	-0.001	133%	-0.001	133%
260	0.006	-0.001	117%	-0.001	117%	-0.001	117%	-0.001	117%
325	0.02	0	100%	-0.001	105%	-0.001	105%	0.001	95%
360	0.033	0.002	94%	0.001	97%	0.002	94%	0.003	91%
390	0.049	0.007	86%	0.005	90%	0.004	92%	0.007	86%
420	0.067	0.012	82%	0.01	85%	0.007	90%	0.012	82%
445	0.093	0.019	80%	0.016	83%	0.011	88%	0.019	80%
460	0.103	0.023	78%	0.02	81%	0.015	85%	0.024	77%

Table 4B.

Salmonella typhimurium				
Time T=0	Control 0.005	73 0.008	%Inhib
0	0	0	
60	-0.001	-0.001	
120	0.001	-0.001	200%
165	0.003	-0.003	200%
230	0.009	-0.004	144%
260	0.013	-0.004	131%
295	0.024	-0.003	113%
325	0.037	-0.002	105%
350	0.051	-0.004	108%
370	0.066	-0.003	105%
390	0.082	0	100%
410	0.098	-0.002	102%
430	0.112	0	100%

Table 4C.

Pseudomonas aeruginosa												
Time *T=0*	Control 0.002	39 0.005	%Inhib	63 0.007	%Inhib	78 0.005	%Inhib					
0	0	0		0		0						
90	0.001	-0.001	200%	0	100%	0	100%					
190	0.002	0	100%	-0.001	150%	0.002	0%					
250	0.003	-0.001	133%	-0.002	167%	0	100%					
300	0.004	-0.001	125%	-0.002	150%	0	100%					
345	0.004	0	100%	0	100%	0	100%					
375	0.005	0.001	80%	0.001	80%	0.002	60%					
415	0.008	0.003	63%	0.004	50%	0.004	50%					
465	0.013	0.008	38%	0.007	46%	0.007	46%					
505	0.02	0.013	35%	0.013	35%	0.011	45%					
545	0.036	0.022	39%	0.022	39%	0.02	44%					
	0.051	0.038	25%	0.034	33%	0.034	33%					
600	0.072	0.055	24%	0.052	28%	0.047	35%					

Table 4D.

Pseudomonas aeruginosa					
Time *T=0*	Control 0	82 0.004	%Inhib	114 0.002	%Inhib
0	0	0		0	
90	0.002	0.001	50%	0.001	50%
120	0.003	0.002	33%	0.003	0%
180	0.006	0.003	50%	0.004	33%
240	0.007	0.004	43%	0.004	43%
305	0.019	0.012	37%	0.011	42%
335	0.024	0.017	29%	0.019	21%
365	0.036	0.027	25%	0.028	22%
400	0.062	0.05	19%	0.049	21%
420	0.074	0.061	18%	0.06	19%
440	0.086	0.074	14%	0.071	17%
460	0.103	0.091	12%	0.087	16%

Table 4E.

Klebsiella pneumoniae							
Time T=0	Control 0.006	114 0.008	%Inhib	78 0.006	%Inhib	73 0.008	%Inhib
0	0	-0.001		0		0	
60	-0.002	-0.002		-0.001		-0.002	
120	0	-0.003		-0.001		-0.0074	
165	0.004	-0.004	200%	-0.003	175%	-0.003	175%
230	0.011	-0.004	136%	-0.001	109%	-0.003	127%
260	0.019	-0.004	200%	0	100%	-0.003	116%
295	0.036	-0.003	108%	0.003	92%	-0.003	108%
325	0.051	-0.001	102%	0.007	86%	-0.003	106%
350	0.064	0	100%	0.012	81%	-0.003	105%
370	0.074	0.002	97%	0.018	76%	-0.003	104%
390	0.088	0.006	93%	0.025	72%	-0.003	103%
410	0.098	0.01	90%	0.037	62%	-0.003	103%

Table 4F.

Klebsiella pneumoniae						
Time T=0	Control 0.006	71 0.009	%Inhib	111 0.008	%Inhib	
0	0	0		0		
60	-0.001	-0.003		-0.002		
135	0.005	0	100%	-0.002	200%	
180	0.012	0	100%	0	100%	
210	0.019	0.004	79%	0.002	83%	
240	0.03	0.006	80%	0.006	67%	
270	0.05	0.014	72%	0.012	63%	
315	0.072	0.03	58%	0.024	54%	
335	0.083	0.039	53%	0.032	54%	
355	0.107	0.051	52%	0.041	51%	

Table 4G.

Yersinia mollaretti								
Time *T=0*	Control 0.003	2 0.002	%Inhib	4 0.002	%Inhib	127 0.002	%Inhib
0	0	0		0		0	
90	0.001	0.001	0%	0.001	0%	0.001	0%
155	0.002	0.002	0%	0.002	0%	0.002	0%
200	0.004	0.003	25%	0.003	25%	0.003	25%
255	0.008	0.003	63%	0.003	63%	0.004	50%
285	0.01	0.004	60%	0.004	60%	0.006	40%
320	0.014	0.008	43%	0.008	43%	0.012	14%
350	0.023	0.012	48%	0.013	43%	0.018	22%
380	0.029	0.018	38%	0.018	38%	0.025	14%
410	0.039	0.026	33%	0.027	31%	0.035	10%
440	0.054	0.035	35%	0.036	33%	0.048	11%
470	0.075	0.05	33%	0.056	25%	0.071	5%
500	0.096	0.07	27%	0.071	26%	0.087	9%
505	0.101	0.072	29%	0.075	26%	0.092	9%

Table 4H.

Yersinia mollaretti					
Time *T=0*	Control 0.002	63 0.004	%Inhib	73 0.004	%Inhib
0	0	0		0	
90	0.001	0.002	-100%	0	100%
190	0.002	0.003	-50%	0.001	50%
250	0.003	0.003	0%	0.001	67%
300	0.003	0	100%	0.001	67%
345	0.006	0.003	50%	0.003	50%
375	0.008	0.005	38%	0.005	38%
415	0.013	0.008	38%	0.009	31%
465	0.023	0.018	22%	0.019	17%
505	0.031	0.027	13%	0.027	18%
545	0.055	0.043	22%	0.043	22%
575	0.074	0.065	12%	0.064	14%
605	0.093	0.083	11%	0.08	14%
615	0.103	0.089	14%	0.088	15%

Table 4I.

Neisseria sicca							
Time *T=0*	Pos. Control OD	16	%Inhib	12	%Inhib	20	%Inhib
	0.029	0.064		0.035		0.084	
0	0	0		0		0	
30	0.002	-0.003		-0.002		-0.002	
65	0.002	-0.003		-0.003		-0.004	
125	0.006	-0.001	117%	-0.002	133%	-0.002	133%
150	0.01	0.001	90%	0	100%	-0.002	120%
180	0.014	0.001	93%	0.002	86%	-0.001	107%
240	0.023	0	100%	0.002	91%	-0.003	113%
300	0.029	0.01	66%	0.009	69%	0.006	79%
330	0.029	0.014	52%	0.013	55%	0.012	59%
390	0.033	0.014	58%	0.009	73%	0.012	64%
450	0.031	0.004	87%	0.009	71%	0.003	90%
490	0.036	0.014	61%	0.008	78%	0.008	78%
520	0.038	0.015	61%	0.014	63%	0.011	71%
560	0.049	0.013	73%	0.002	96%	0.007	86%
590	0.052	0.017	67%	0.014	73%	0.012	77%
620	0.057	0.018	68%	0.014	75%	0.014	75%
650	0.059	0.016	73%	0.018	69%	0.014	76%
680	0.063	0.018	71%	0.016	75%	0.016	75%
710	0.068	0.019	72%	0.017	75%	0.016	76%

Table 4J.

Neisseria sicca					
Time *T=0*	Pos. Control OD 0.029	3 0.018	%Inhib	15 0.056	%Inhib
0	0	0		0	
30	0.002	-0.001		0.001	
65	0.002	-0.004		0	
125	0.006	-0.001	117%	0.002	67%
150	0.01	0.004	60%	-0.003	130%
180	0.014	0.005	64%	-0.002	114%
240	0.025	0.004	84%	-0.003	112%
300	0.027	0.008	70%	0.01	63%
330	0.029	0.015	48%	0.018	38%
390	0.033	0.012	64%	-0.003	109%
450	0.031	0.005	84%	0.01	68%
490	0.036	0.012	67%	0.016	56%
520	0.038	0.007	82%	0.018	53%
560	0.049	0.011	78%	0.021	57%
590	0.052	0.011	79%	0.02	62%
620	0.057	0.011	81%	0.018	68%
650	0.059	0.012	80%	0.018	69%
680	0.063	0.011	83%	0.02	68%
710	0.068	0.012	82%	0.17	75%

Table 4K.

Serratia liquefaciens									
Time *T=0*	Control -0.001	2 -0.001	%Inhib	39 0.001	%Inhib	82 0.002	%Inhib	114 0	%Inhib
0	0	0		0		0		0	
110	0.002	0.002	0%	0.002	0%	0	100%	0.002	0%
180	0.003	0.003	0%	0.001	67%	0.001	67%	0.002	33%
240	0.003	0.002	33%	0.001	67%	0.001	67%	0.002	33%
300	0.002	0.002	0%	0.001	50%	0	100%	0.001	50%
360	0.005	0.002	60%	0.001	80%	0	100%	0.001	80%
420	0.011	0.003	73%	0.001	91%	0.001	91%	0.002	82%
475	0.022	0.003	86%	0.002	91%	0.001	95%	0.003	86%
520	0.041	0.003	93%	0.001	98%	0.001	98%	0.002	95%
610	0.082	0.003	96%	0.001	99%	0.001	99%	0.002	98%
655	0.1	0.003	97%	0.001	99%	0.001	99%	0.002	98%

Table 4L.

Streptococcus mutans								
Time *T=0*	Control 0.184	1 0.187	%Inhib	89 0.187	%Inhib	127 0.187	%Inhib
0	0	0		0		0	
60	0.001	-0.003	400%	-0.001	200%	-0.002	300%
115	0.006	-0.001	117%	0.003	50%	0.001	83%
145	0.011	-0.001	109%	0.003	73%	0.003	73%
180	0.016	0.002	88%	0.008	50%	0.006	63%
210	0.022	0.004	82%	0.01	55%	0.008	64%
245	0.031	0.009	71%	0.015	52%	0.014	55%
290	0.047	0.015	68%	0.021	55%	0.021	55%
320	0.059	0.022	63%	0.026	56%	0.03	49%
350	0.071	0.03	58%	0.032	55%	0.04	44%
385	0.082	0.036	56%	0.032	61%	0.047	43%
415	0.097	0.042	57%	0.036	63%	0.05	48%
445	0.109	0.045	59%	0.039	64%	0.063	42%

Table 4M.

Streptococcus mutans					
Time *T=0*	Control 0.184	132 0.187	%Inhib	114 0.183	%Inhib
0	0	0		0	
60	0.001	-0.002	300%	-0.003	400%
115	0.006	0.001	83%	-0.001	117%
145	0.011	0.001	91%	0.002	82%
180	0.006	0.006	63%	0.004	75%
210	0.022	0.008	64%	0.008	64%
245	0.031	0.01	68%	0.013	58%
290	0.047	0.017	64%	0.025	47%
320	0.059	0.022	63%	0.034	42%
350	0.071	0.027	62%	0.045	37%
385	0.082	0.028	66%	0.054	34%
415	0.097	0.033	66%	0.062	36%
445	0.109	0.034	69%	0.069	37%

Table 4N.

Streptococcus pyogenes									
Time T=0	Control 0.177	1 0.179	%Inhib	89 0.177	%Inhib	127 0.179	%Inhib		
0	0	0		0		0			
110	0.001	0	100%	-0.001	200%	-0.004	500%		
170	0.003	-0.002	167%	-0.002	167%	-0.005	267%		
210	0.005	-0.001	120%	0	100%	-0.003	160%		
240	0.008	-0.001	113%	-0.001	113%	-0.002	125%		
300	0.01	0	100%	0.001	90%	0	100%		
345	0.014	0.003	79%	0.002	86%	0	100%		
390	0.021	0.006	71%	0.003	86%	0	100%		
450	0.036	0.01	72%	0.008	78%	0.007	81%		
510	0.067	0.017	75%	0.015	78%	0.015	78%		
540	0.093	0.025	73%	0.026	72%	0.026	72%		
555	0.107	0.028	74%	0.029	73%	0.025	77%		

Table 4O.

Streptococcus pyogenes						
Time T=0	Control 0.177	132 0.177	%Inhib	114 0.181	%Inhib	
0	0	0		0		
110	0.001	-0.001	200%	-0.001	133%	
170	0.003	-0.003	200%	-0.003	175%	
210	0.005	0	100%	-0.004	200%	
240	0.008	-0.001	113%	-0.001	117%	
300	0.01	0.001	90%	0	100%	
345	0.014	0.002	86%	0.001	91%	
390	0.021	0.004	81%	0.005	69%	
450	0.036	0.009	75%	0.015	55%	
510	0.067	0.015	78%	0.031	47%	
540	0.093	0.021	77%	0.047	45%	
555	0.107	0.021	80%	0.053	48%	

Table 4P.

Shigella							
Time *T=0*	Control 0.001	1 0.003	%Inh	89 0.003	%Inh	127 0.004	%Inh
0	0	0		0		0	
95	0.001	-0.001	200%	-0.001	200%	-0.001	200%
155	0.005	-0.001	120%	-0.003	160%	-0.002	140%
215	0.009	-0.001	111%	-0.002	122%	-0.002	122%
275	0.027	0	100%	-0.002	107%	-0.001	104%
305	0.038	0	100%	-0.003	108%	-0.002	105%
335	0.044	0.001	98%	-0.001	102%	-0.003	107%
365	0.047	0.004	91%	-0.002	104%	-0.001	102%
395	0.051	0.006	88%	-0.002	104%	-0.001	102%
425	0.051	0.008	84%	-0.003	106%	-0.001	102%

Table 4Q.

Shigella					
Time *T=0*	Control 0.001	132 0.003	%Inh	114 0.003	%Inh
0	0	0		0	
95	0.001	-0.001	200%	-0.001	200%
155	0.005	-0.001	120%	-0.002	140%
215	0.009	-0.001	111%	-0.003	133%
275	0.027	-0.001	104%	-0.003	111%
305	0.038	-0.002	105%	-0.003	108%
335	0.044	-0.003	107%	-0.003	107%
365	0.047	-0.001	102%	-0.003	106%
395	0.051	0	100%	-0.002	104%
425	0.051	0	100%	-0.002	104%

Table 4R.

Haemophilus			
Time *T=0*	Control 0.161	78 0.017	%Inh
0	0	0	
70	0.007	0	100%
140	0.012	0.008	33%
190	0.013	0.01	23%
235	0.013	0.013	0%
275	0.013	0.013	0%
305	0.015	0.012	20%
365	0.016	0.013	19%
24'	0.026	0.011	58%
29' 50'	0.051	0.014	73%
46'	0.241	0.021	91%

Table 4S.

Mycobacterium							
Time *T=0*	Control 0.167	114 0.163	%Inh	10 0.168	%Inh	21 0.165	%Inh
0	0	0		0		0	
90	0.006	0.001	83%	0.001	83%	0.002	67%
120	0.009	0.003	67%	0.002	78%	0.006	33%
165	0.014	0.005	64%	0.005	64%	0.01	29%
195	0.021	0.006	71%	0.005	76%	0.008	62%
240	0.021	0.007	67%	0.007	67%	0.009	57%
270	0.018	0.013	28%	0.01	44%	0.013	28%
305	0.028	0.016	43%	0.012	57%	0.014	50%
405	0.04	0.026	35%	0.032	20%	0.025	38%
465	0.051	0.032	37%	0.041	20%	0.032	37%
525	0.063	0.04	37%	0.051	19%	0.043	32%
555	0.073	0.046	37%	0.06	18%	0.052	29%
585	0.08	0.051	36%	0.065	19%	0.055	31%
615	0.085	0.062	27%	0.073	14%	0.062	27%
645	0.097	0.065	33%	0.079	19%	0.068	30%

Table 4T.

Mycobacterium					
Time *T=0*	Control 0.167	18 0.163	%Inh	78 0.166	%Inh
0	0	0		0	
90	0.006	-0.001	117%	0	100%
120	0.009	0.002	78%	0.003	67%
165	0.014	0.007	50%	0.003	79%
195	0.021	0.006	71%	0.004	81%
240	0.021	0.008	62%	0.006	71%
270	0.018	0.008	56%	0.003	83%
305	0.028	0.01	64%	0.009	68%
405	0.04	0.022	45%	0.018	55%
465	0.051	0.03	41%	0.024	53%
525	0.063	0.037	41%	0.029	54%
555	0.073	0.044	40%	0.037	49%
585	0.08	0.047	41%	0.04	50%
615	0.085	0.052	39%	0.042	51%
645	0.097	0.059	39%	0.056	42%

Table 4U.

Helicobacter			
Time *T=0*	Control 0.08	78 0.084	%Inh
0	0	0	
70	-0.004	-0.009	
140	0	-0.006	
190	0.001	-0.005	600%
235	0.003	-0.001	133%
275	0.004	0	100%
305	0.009	0.004	56%
365	0.01	0.003	70%
24'	0.057	0.01	82%
29' 50'	0.065	0.012	82%
45'	0.065	0.005	92%

Table 4V.

Enterococcus									
Time T=0	Control 0.09	89 0.088	%Inh	127 0.087	%Inh	132 0.088	%Inh	p127 0.086	%Inh
0	0	0		0		0		0	
60	0	-0.004		-0.006		-0.006		-0.007	
105	0.005	-0.004	180%	-0.002	140%	-0.003	160%	-0.005	200%
150	0.026	0.008	69%	0.009	65%	0.008	69%	0.01	62%
170	0.066	0.029	56%	0.029	56%	0.025	62%	0.032	52%
195	0.076	0.04	47%	0.04	47%	0.036	53%	0.043	43%
210	0.091	0.051	44%	0.052	43%	0.047	48%	0.054	41%
215	0.11	0.062	44%	0.064	42%	0.055	50%	0.066	40%

Table 4W.

Enterococcus					
Time T=0	Control 0.042	1 0.049	%Inh	76 0.049	%Inh
0	0	0		0	
60	0.002	-0.002	200%	-0.001	150%
120	0.006	-0.001	117%	0	100%
160	0.023	0.002	91%	0.003	87%
190	0.036	0.01	72%	0.013	64%
210	0.051	0.015	71%	0.02	61%
230	0.074	0.031	58%	0.04	46%
245	0.083	0.037	55%	0.046	45%
255	0.094	0.047	50%	0.057	39%
265	0.109	0.054	50%	0.065	40%

Table 4X.

Streptococcus pneumonia								
Time T=0	Control 0.17	1 0.172	%Inh	78 0.174	%Inh	114 0.17	%Inh
0	0	0		0		0	
60	0.004	0	100%	0	100%	-0.001	125%
110	0.003	-0.005	267%	-0.001	133%	-0.001	133%
170	0.003	-0.003	200%	-0.001	133%	-0.001	133%
220	0.004	-0.002	150%	0	100%	-0.001	125%
260	0.004	-0.001	125%	-0.001	125%	-0.001	125%
310	0.007	-0.002	129%	0	100%	-0.001	114%
370	0.008	-0.003	138%	0	100%	0	100%
445	0.009	-0.002	122%	0	100%	0	100%
485	0.009	-0.003	133%	0.001	89%	0.001	89%
19'35'	0.014	0.001	94%	0.011	21%	0.008	43%
21'35'	0.014	0.001	93%	0.01	29%	0.006	57%
23'35'	0.015	0.002	87%	0.012	20%	0.008	47%
27'	0.016	0.001	94%	0.013	19%	0.009	44%
28'30'	0.016	0.002	88%	0.014	12%	0.01	38%
45'20'	0.023	0.018	22%	0.024	-4%	0.018	22%
48'20'	0.024	0.008	67%	0.025	-4%	0.014	42%
51'20'	0.024	0.01	58%	0.035	-46%	0.022	8%
54'20'	0.026	0.011	58%	0.028	-8%	0.021	19%
70'35'	0.035	0.014	60%	0.033	6%	0.027	23%
95'35'	0.05	0.025	50%	0.059	-18%	0.04	20%
101'	0.068	0.025	63%	0.046	32%	0.043	37%

Table 4Y.

Streptococcus pneumonia					
Time T=0	Control 0.17	127 0.172	%Inh	132 0.167	%Inh
0	0	0		0	
60	0.004	-0.001	125%	-0.001	125%
110	0.003	-0.001	133%	-0.003	200%
170	0.003	-0.002	167%	-0.003	200%
220	0.004	-0.002	150%	-0.002	150%
260	0.004	-0.001	125%	-0.002	150%
310	0.007	-0.002	129%	-0.001	114%
370	0.008	0	100%	0	100%
445	0.009	0	100%	0	100%
485	0.009	0	100%	0	100%
19'35'	0.014	0.008	43%	0.009	36%
21'35'	0.014	0.007	50%	0.009	36%
23'35'	0.015	0.008	47%	0.009	40%
27'	0.016	0.01	37%	0.013	19%
28'30'	0.016	0.012	25%	0.012	25%
45'20'	0.023	0.019	17%	0.022	4%
48'20'	0.024	0.2	17%	0.021	13%
51'20'	0.024	0.021	12%	0.022	8%
54'20'	0.026	0.022	15%	0.024	8%
70'35'	0.035	0.027	23%	0.033	6%
95'35'	0.05	0.048	4%	0.05	0%
101'	0.068	0.048	29%	0.052	24%

Table 4Z.

Vibrio					
Time T=0	Control 0.138	78 0.142	%Inh	127 0.143	%Inh
0	0	0		0	
70	0.002	-0.001	150%	-0.003	250%
140	0.002	0	100%	-0.002	200%
190	0.005	0	100%	0	100%
235	0.005	0.001	80%	-0.002	140%
275	0.005	0.001	80%	-0.003	160%
305	0.005	0	100%	0	100%
365	0.004	-0.001	125%	-0.002	150%
24'	0.006	0.003	50%	0	100%
46'	0.177	0.006	97%	0.129	27%

Table 5A.

Staphylococcus aureus 13301									
Time T=0	Control 0.001	21 0.004	%Inhib	68 0.002	%Inhib	85 0.002	%Inhib
0	0	0		0		0	
65	0.001	0.001	0%	0.002	-100%	0.001	0%
125	0.002	0.002	0%	0.003	-50%	0.002	0%
185	0.003	0.002	33%	0.003	0%	0.003	0%
240	0.003	0.002	33%	0.003	0%	0.002	33%
295	0.004	0.001	75%	0.003	25%	0.002	50%
340	0.007	0.002	71%	0.003	57%	0.003	57%
385	0.011	0.004	64%	0.003	73%	0.002	82%
415	0.016	0.002	88%	0.003	81%	0.001	94%
445	0.021	0.002	90%	0.003	86%	0.002	90%
475	0.032	0.002	94%	0.003	91%	0.002	94%
505	0.029	0.001	97%	0.003	90%	0.002	93%
535	0.045	0.001	98%	0.003	93%	0.002	96%
565	0.057	0	100%	0.001	98%	0.003	95%
595	0.072	0.002	97%	0.003	96%	0.003	96%
625	0.09	0.002	98%	0.002	98%	0.002	98%
25'	0.456	-0.002	100%	0	100%	0.026	94%

Table 5B.

Staphylococcus aureus 13301					
Time *T=0*	Control 0.001	112 0.005	%Inhib	18 0.003	%Inhib
0	0	0		0	
65	0.001	0	100%	0.001	0%
125	0.002	0.002	0%	0.003	-50%
185	0.003	0.001	67%	0.002	33%
240	0.003	0.001	67%	0.002	33%
295	0.004	0.002	50%	0.002	50%
340	0.007	0.001	86%	0.003	57%
385	0.011	0.001	91%	0.003	73%
415	0.016	0	100%	0.002	88%
445	0.021	0	100%	0.002	90%
475	0.032	0.001	97%	0.002	94%
505	0.029	0.001	97%	0.002	93%
535	0.045	0.002	96%	0.002	96%
565	0.057	0.002	96%	0.002	96%
595	0.072	0.001	99%	0.002	97%
625	0.09	0	100%	0.002	98%
25'	0.456	-0.003	101%	0	100%

Table 5C.

Escherichia coli 35218 Multiple Drug Resistance							
Time *T=0*	Control 0.001	21 0.004	%Inhib	68 0.005	%Inhib	85 0.005	%Inhib
0	0	0		0.001		0.001	
70	0.002	0.001	50%	-0.002	200%	-0.001	150%
130	0.002	0.001	50%	-0.001	150%	-0.001	150%
190	0.002	-0.001	150%	-0.003	250%	-0.002	200%
250	0.009	-0.002	122%	-0.003	133%	-0.003	133%
295	0.015	-0.002	113%	-0.002	113%	-0.002	113%
325	0.024	-0.001	104%	-0.002	108%	-0.002	108%
355	0.032	-0.002	106%	-0.002	106%	-0.002	106%
385	0.046	-0.002	104%	-0.003	107%	-0.002	104%
415	0.068	-0.001	101%	-0.002	103%	-0.002	103%
445	0.087	-0.001	101%	-0.001	101%	-0.001	101%
465	0.1	-0.001	101%	-0.001	101%	-0.002	102%
555	0.138	0.009	93%	0.01	93%	0.005	96%
27'	0.191	0.196	-3%	0.192	-1%	0.192	-1%

Table 5D.

Escherichia coli 35218 Multiple Drug Resistance					
Time T=0	Control 0.001	112 0.004	%Inhib	18 0.003	%Inhib
0	0	-0.002		0	
70	0.002	-0.004	300%	-0.001	150%
130	0.002	-0.005	350%	-0.001	150%
190	0.002	-0.005	350%	-0.001	150%
250	0.009	-0.005	156%	-0.001	111%
295	0.015	-0.004	127%	0	100%
325	0.024	-0.004	117%	-0.001	104%
355	0.032	-0.005	116%	0	100%
385	0.046	-0.004	109%	-0.001	102%
415	0.068	-0.004	106%	0	100%
445	0.087	-0.003	103%	0.003	97%
465	0.1	-0.004	104%	0.004	96%
555	0.138	0.008	94%	0.026	81%
27'	0.191	0.178	7%	0.174	9%

Table 5E.

Escherichia coli 25922 NBT89 At different concentrations							
Time T=0	Control 0	2.1mg 0.004	%Inh	1.05mg 0.003	%Inh	0.525mg 0.002	%Inhib
0	0	0		0		0	
60	0.001	-0.001	200%	-0.001	200%	-0.001	200%
120	0.001	-0.002	100%	-0.001	200%	-0.001	200%
225	0.005	-0.002	140%	-0.001	120%	-0.001	120%
270	0.012	-0.001	208%	-0.001	108%	-0.001	108%
315	0.027	-0.001	104%	-0.001	104%	0	100%
335	0.035	-0.001	103%	-0.001	103%	0.001	97%
355	0.044	-0.002	105%	-0.001	102%	0.002	95%
315	0.052	-0.002	104%	-0.001	102%	0.002	96%
395	0.06	-0.003	105%	-0.001	102%	0.002	97%
415	0.081	-0.002	102%	-0.001	101%	0.003	96%
430	0.092	-0.002	102%	-0.001	101%	0.005	95%
445	0.101	-0.002	102%	0.000	100%	0.009	91%
24 hr			27%		18%		16%

Table 5f

Escherichia coli 25922 NBT89 At different concentrations

Time T=0	Control 0	0.265mg 0.001	%Inhib	0.133mg 0	%Inhib	0.07mg 0.002	%Inhib	.035mg 0.001	%Inhib
0	0	0		0		0		0	
60	0.001	-0.001	200%	0	100%	0	100%	0	100%
120	0.005	-0.001	200%	0	100%	-0.001	100%	0	100%
225	0.005	-0.001	120%	0	100%	0.001	80%	0.002	60%
270	0.013	-0.001	108%	0.003	75%	0.004	67%	0.006	50%
315	0.027	0.003	89%	0.01	63%	0.012	59%	0.029	34%
315	0.035	0.004	89%	0.015	57%	0.018	49%	0.022	37%
355	0.044	0.006	86%	0.021	52%	0.024	57%	0.029	34%
375	0.052	0.008	85%	0.025	52%	0.029	44%	0.035	33%
395	0.06	0.012	80%	0.032	47%	0.037	38%	0.044	27%
415	0.081	0.018	78%	0.044	46%	0.052	36%	0.061	25%
430	0.092	0.021	77%	0.054	41%	0.063	32%	0.072	22%
445	0.101	0.028	72%	0.064	37%	0.073	28%	0.082	19%
24 hr			14%		11%		15%		11%

Table 6A
The Effects of Oligonucleotide Purification Method on the Percent Inhibition of *Escherichia coli* 35218 (See Section 5.5.)

Time	Control	A	B	C	D	E
0	0					
90	.003	100%	100%	100%	100%	100%
150	.004	100%	100%	100%	100%	100%
220	.008	75%	100%	100%	63%	100%
270	.014	36%	100%	100%	14%	100%
315	.029	38%	100%	100%	10%	100%
345	.038	21%	100%	100%	8%	100%
375	.059	25%	93%	97%	3%	100%
400	.079	27%	90%	90%	6%	99%
420	.089	25%	84%	84%	5%	98%
435	.099	24%	83%	83%	4%	96%

Table 6B
The Effects of Oligonucleotide Purification Method on the Percent Inhibition of *Escherichia coli* 35218 (See Section 5.5.)

Time	Control	F	G	H	I
0	0				
90	.003	100%	100%	100%	100%
150	.004	100%	100%	100%	100%
220	.008	100%	100%	100%	100%
270	.014	100%	100%	100%	100%
315	.029	63%	100%	100%	100%
345	.088	47%	100%	100%	100%
375	.059	50%	100%	98%	100%
400	.079	34%	96%	91%	100%
420	.089	43%	96%	88%	100%
435	.099	41%	93%	86%	100%

Table 7
Antigene Oligonucleotides Targeted to DNA sense strand for Triplex Formation

Escherichia coli 35218 Multiple Drug Resistance					
Time T=0	Control 0.002	96.SS 0.008	%Inhib	73.SS 0.004	%Inhib
0	0	0.004		0	
60	0.001	0	100%	0.001	0%
120	0.001	0	100%	0	100%
120	0.001	0	100%	-0.001	200%
240	0.005	-0.001	120%	0	100%
285	0.012	-0.001	108%	-0.002	117%
350	0.027	-0.001	104%	0	100%
390	0.043	0.002	95%	0.001	98%
420	0.063	0.006	90%	0.004	94%
450	0.082	0.01	88%	0.008	90%
470	0.096	0.017	82%	0.01	90%
500	0.106	0.023	78%	0.012	89%

Table 8A.

Escherichia coli 11170 Streptomycin Resistant			
Time *T=0*	Control 0	73 0.004	%Inhib
0	0	0	
60	0.005	0	100%
140	0.011	-0.002	118%
170	0.013	0	100%
215	0.021	0.003	86%
245	0.032	0.005	84%
275	0.045	0.007	84%
305	0.062	0.009	85%
325	0.076	0.009	88%
340	0.09	0.01	89%
350	0.1	0.012	88%

Table 8B.

Escherichia coli 29214 Sulfonamide Resistant			
Time *T=0*	Control 0.001	73 0.003	%Inhib
0	0	0	
50	0.001	-0.002	300%
130	0.005	-0.001	120%
175	0.015	-0.001	107%
205	0.022	-0.001	105%
235	0.031	-0.001	103%
270	0.05	0	100%
295	0.065	0	100%
315	0.081	0.003	96%
335	0.092	0.006	93%

Table 8C.

Escherichia coli 25922 Intermediate Penicillin Resistant			
Time T=0	Control 0.004	73 0.006	%Inhib
0	0	0	
60	-0.00 1	-0.001	
230	0	-0.001	
165	0	-0.001	
230	0.003	-0.001	133%
260	0.005	-0.002	140%
365	0.014	-0.002	114%
335	0.021	-0.002	103%
365	0.033	-0.002	106%
395	0.052	-0.001	102%
415	0.066	-0.002	103%
435	0.08	-0.002	103%
455	0.093	-0.002	102%
475	0.108	-0.001	101%

Table 8D.

Salmonella typhimurium 23564			
Time T=0	Control 0.005	73 0.008	%Inhib
0	0	0	
60	0.001	-0.001	
120	0.001	-0.001	200%
165	0.003	-0.003	200%
230	0.009	-0.004	144%
260	0.013	-0.004	131%
295	0.024	-0.003	113%
325	0.037	-0.002	105%
350	0.051	-0.004	108%
370	0.066	-0.003	105%
390	0.082	0	100%
410	0.098	-0.002	102%

Table 8E.

Klebsiella pneumoniae 4352			
Time *T=0*	Control 0.006	73 0.008	%Inhib
0	0	0	
60	-0.002	-0.002	
120	0	-0.0074	
165	0.004	-0.003	175%
230	0.011	-0.003	127%
260	0.019	-0.003	116%
295	0.036	-0.003	108%
325	0.051	-0.003	106%
350	0.064	-0.003	105%
370	0.074	-0.003	104%
390	0.088	-0.003	103%
410	0.098	-0.003	103%

Table 8F.

Escherichia coli 35218 Multiple Drug Resistance			
Time *T=0*	Control 0.001	73 -0.004	%Inhib
0	0	0	
60	0.001	-0.003	400%
120	0.003	-0.002	167%
180	0.013	-0.001	108%
180	0.019	-0.002	111%
180	0.027	-0.001	104%
270	0.04	0	100%
300	0.058	0.003	95%
320	0.075	0.006	92%
340	0.089	0.008	91%
355	0.103	0.013	87%

Table 8G.

Staphylococcus aureus 29213			
Time *T=0*	Control 0	73 -0.007	%Inhib
0	0	0	
60	0	-0.003	
120	0.003	-0.004	233%
165	0.006	-0.003	150%
210	0.01	0.001	90%
240	0.014	0.004	71%
270	0.024	0.011	54%
300	0.034	0.021	38%
340	0.48	0.033	31%
360	0.06	0.041	32%
380	0.072	0.05	31%
400	0.09	0.062	31%
420	0.102	0.07	31%

Tables 9(A-G)
Oligonucleotide NBT 114 vs. Different Strains of Bacteria

Table 9A.

Escherichia coli 11370 Streptomycin Resistant			
Time T=0	Control 0	114 0.004	% Inhib
0	0	0	
60	0.005	-0.003	160%
140	0.011	0	100%
170	0.013	0.003	77%
215	0.021	0.009	57%
245	0.032	0.014	56%
275	0.045	0.018	60%
305	0.062	0.024	61%
325	0.076	0.03	61%
340	0.09	0.034	62%
350	0.1	0.036	64%

Table 9B.

Escherichia coli 29214 Sulfonamide Resistant			
Time T=0	Control 0.001	114 0.003	% Inhib
0	0	0	
60	0.001	-0.002	300%
130	0.005	-0.001	120%
175	0.015	-0.001	107%
205	0.022	-0.001	105%
235	0.031	0	100%
270	0.05	0.005	90%
295	0.065	0.007	89%
315	0.081	0.012	85%
335	0.092	0.017	82%

Table 9C.

Escherichia coli 25922 Intermediate Penicillin Resistant			
Time T=0	Control 0.004	114 0.008	% Inhib
0	0	0	
60	-0.001	0	
120	0	-0.002	
295	0	-0.004	
230	0.003	-0.004	233%
260	0.005	-0.004	180%
305	0.014	-0.002	114%
335	0.021	0	100%
365	0.033	0.001	97%
395	0.052	0.007	87%
415	0.066	0.012	82%
435	0.08	0.018	78%
455	0.037	0.026	72%
475	0.108	0.035	88%

Table 9D.

Salmonella typhimurium 23564			
Time T=0	Control 0.005	114 0.007	% Inhib
0	0	0	
60	-0.001	0	
120	0.001	-0.001	200%
165	0.003	-0.003	200%
230	0.009	-0.003	133%
260	0.013	-0.002	115%
295	0.037	0	100%
325	0.037	0.003	92%
350	0.051	0.009	82%
370	0.066	0.012	82%
390	0.082	0.017	79%
410	0.098	0.024	76%

Table 9E.

Klebsiella pneumoniae 4352			
Time *T=0*	Control *0.006*	114 *0.008*	% Inhib
0	0	-0.001	
60	-0.002	-0.002	
120	0	-0.003	
165	0.004	-0.004	200%
230	0.011	-0.004	136%
260	0.019	-0.004	121%
295	0.036	-0.003	108%
325	0.051	-0.001	102%
350	0.064	0	100%
370	0.074	0.002	97%
390	0.088	0.006	93%
410	0.098	0.01	90%

Table 9F.

Escherichia coli 35218 Multiple Drug Resistance			
Time *T=0*	Control 0.001	114 0.003	% Inhib
0	0	0	
60	0.001	-0.002	300%
120	0.003	-0.001	133%
180	0.013	0	100%
210	0.019	0	100%
240	0.027	0.002	93%
270	0.04	0.006	85%
300	0.058	0.014	76%
320	0.075	0.023	69%
340	0.089	0.031	65%
355	0.103	0.04	61%

Table 9G.

Staphylococcus aureus 29213			
Time T=0	Control 0	114 0.005	% Inhib
0	0	0	
60	0	-0.003	
120	0.003	-0.003	200%
165	0.006	-0.002	133%
210	0.01	0.002	80%
240	0.014	0.005	64%
270	0.024	0.012	50%
300	0.034	0.019	44%
340	0.048	0.031	35%
360	0.06	0.039	35%
380	0.072	0.047	35%
400	0.09	0.058	36%
420	0.102	0.063	38%

Table 10

Restoration of Ampicillin Sensitivity in an Ampicillin Resistant Strain of *Escherichia coli* Y1088						
Time T=0	Control +50 µg/ml amp	NBT 14 +50µg/ml amp	%Inhib	Control +250µg/ml amp	NBT 14 +250µg/ml amp	%Inhib
0	0	0		0	0	
60	0	0		0	0	
120	0	0		0	0	
180	0	0		0	0	
245	0	0		0.002	0	
270	0	0		0.004	0.001	75%
290	0.001	0.001		0.006	0.002	67%
310	0.006	0.002	67%	0.007	0.002	71%
330	0.007	0.003	57%	0.013	0.004	69%
355	0.013	0.005	61%	0.02	0.006	70%
370	0.017	0.007	59%	0.022	0.008	64%
390	0.026	0.011	58%	0.03	0.013	57%
410	0.087	0.016	50%	0.039	0.018	54%
430	0.038	0.021	45%	0.043	0.023	46%
450	0.052	0.026	50%	0.062	0.031	50%
470	0.069	0.035	49%	0.075	0.041	45%

Table 11

Number of Bacteria in the blood

	T=0	T=24 hr.	Change over 24 hours
Saline Control	1x10⁶ bacteria	3x10⁶ bacteria	3 fold increase in bacteria
+Oligo NBT 132	1x10⁶ bacteria	0.13x10⁶ bacteria	10 fold reduction in bacteria

What is claimed is:

1. A method for treating an animal, including a human, having an infection caused by a pathogenic bacterium, comprising: administering to the animal a composition comprising a pharmaceutically acceptable carrier and a substantially nuclease resistant oligonucleotide having about 8 to about 80 nucleotides and targeted to a nucleic acid or protein in the bacterium in an amount sufficient to alleviate a symptom of the infection.

2. The method of claim 1, wherein the nucleic acid or protein is involved in the synthesis, metabolism, assembly or regulation of at least one of the group consisting of energy, DNA replication, cell division, regulatory proteins, cell walls, sugars, virulence, fatty acids, mRNAs, tRNAs, rRNAs, ribosomal proteins, proteins involved in protein synthesis, phospholipids, periplasmic proteins, secretory proteins, flagellar proteins, transport proteins, amino acids, lipopolysaccharides, purines, pyrimidines, pili, outer membrane proteins, nitrogen, antibiotic binding proteins and vitamins.

3. The method of claim 1, wherein the oligonucleotide is capable of associating with a nucleic acid or protein in the bacterium such that it inhibits at least one of the group consisting of bacterial growth, reproduction, metabolism, synthesis of toxins, progress of infection and virulence.

4. The method of claim 3, wherein the associating is hybridizing to an mRNA in the bacterium at or near the initiation codon, in the 5' untranslated region, in the 3' untranslated region, internal to the coding region or an intermediate region of the mRNA.

5. The method of claim 3, wherein the associating is hybridizing to DNA in the bacterium.

6. The method of claim 5, wherein the hybridizing forms a triplex structure.

7. The method of claim 3, wherein the associating is binding with a protein in the bacterium.

8. The method of claim 1, wherein the oligonucleotide hybridizes to any one of the operons listed in Table 1.

9. The method of claim 1, wherein the oligonucleotide hybridizes to any one of the genes listed in Table 1.

10. The method of claim 1, wherein the oligonucleotide comprises a sequence drawn from SEQ ID NOS. 1-176 of the Sequence Listing or a functional equivalent thereof.

11. The method of claim 1, wherein the oligonucleotide has been purified by a method comprising at least one method from the group consisting of diafiltration, gel filtration, high performance liquid chromatography, fast performance liquid chromatography, alcohol precipitations, or alcohol extractions followed by ethanol or chloroform extractions.

12. The method of claim 1, wherein the oligonucleotide was purified by gel filtration.

13. The method of claim 1, wherein the oligonucleotide is capable of inhibiting growth of the bacterium in an MIC assay.

14. The method of claim 1, wherein the oligonucleotide has been modified in at least one base, sugar or internucleotide linkage so as to increase nuclease resistance, stability, specificity or uptake by bacteria of the oligonucleotide.

15. The method of claim 1, wherein the oligonucleotide is selected from at least one of the group consisting of:

a) partially or fully substituted phosphorothioate oligonucleotides or analogues thereof;

b) partially or fully substituted alkyl phosphonate oligonucleotides or analogues thereof;

c) partially or fully substituted phosphate ester oligonucleotides or analogues thereof;

d) partially or fully substituted phosphoramidate oligonucleotides or analogues thereof;

e) partially or fully substituted 2' modified RNA oligonucleotides or analogues thereof;

f) partially or fully substituted morpholino oligonucleotides or analogues thereof;

g) partially or fully substituted peptide nucleic acid oligonucleotides or analogues thereof;

h) partially or fully substituted dithioate oligonucleotides or analogues thereof;

i) partially or fully substituted 5' thio oligonucleotides or analogues thereof;

j) partially or fully substituted propyne oligonucleotides or analogues thereof;

k) chimerics of any combination of the above; and

l) any chemical modifications of the oligonucleotide which leave the oligonucleotide capable of specifically binding the nucleic acid or protein.

16. The method of claim 1, wherein the administration is selected from the group consisting of oral, intravenous, intramuscular, intraperitoneal, subcutaneous, intradermal, inhalation and topical administration.

17. The method of claim 1, wherein the bacterium is gram positive.

18. The method of claim 1, wherein the bacterium is gram negative.

19. The method of claim 1, wherein the bacterium is acid fast.

20. The method of claim 1, wherein the bacterium is a member of a genus selected from the group consisting of *Aerococcus, Listeria, Streptomyces, Actinomadura, Lactobacillus, Eubacterium, Arachnia, Mycobacterium, Peptostreptococcus, Staphylococcus, Corynebacterium, Erysipelothrix, Dermatophilus, Rhodococcus, Bifodobacterium, Lactobacillus, Streptococcus, Bacillus, Peptococcus, Micrococcus, Kurthia, Nocardia, Nocardiopsis, Rothia, Propionibacterium, Actinomyces, Enterococcus, Pneumococcus,* and *Clostridia*.

21. The method of claim 1, wherein the bacterium is a member of the genus *Staphylococcus*.

22. The method of claim 21, wherein the bacterium is *Staphylococcus aureus*.

23. The method of claim 1, wherein the bacterium is a member of the genus *Pseudomonas*.

24. The method of claim 1, wherein the bacterium is a member of the genus *Klebsiella*.

25. The method of claim 1, wherein the bacterium is a member of the genus *Yersinia*.

26. The method of claim 1, wherein the bacterium is a member of the genus *Neisseria*.

27. The method of claim 1, wherein the bacterium is a member of the genus *Serratia*.

28. The method of claim 1, wherein the bacterium is a member of the genus *Streptococcus*.

29. The method of claim 28, wherein the bacterium is *Streptococcus pyogenes*.

30. The method of claim 28, wherein the bacterium is *Streptococcus pneumoniae*.

31. The method of claim 1, wherein the bacterium is a member of the genus *Shigella*.

32. The method of claim 1, wherein the bacterium is a member of the genus *Haemophilus*.

33. The method of claim 1, wherein the bacterium is a member of the genus *Mycobacterium*.

34. The method of claim 1, wherein the bacterium is a member of the genus *Helicobacter*.

35. The method of claim 1, wherein the bacterium is a member of the genus *Enterococcus*.

36. The method of claim 1, wherein the bacterium is a member of the genus *Vibrio*.

37. The method of claim 1, wherein the bacterium is a member of the genus *Salmonella*.

38. The method of claim 1, wherein the bacterium is a *Pneumococcus*.

39. The method of claim 1, wherein the bacterium is *Escherichia coli*.

40. A composition comprising a pharmaceutically acceptable carrier and a substantially nuclease resistant oligonucleotide having about 8 to about 80 nucleotides and

targeted to a nucleic acid or protein in the bacterium in an amount sufficient to alleviate a symptom of the infection.

41. The composition of claim 40, wherein the nucleic acid or protein is involved in the synthesis, metabolism, assembly or regulation of at least one of the group consisting of energy, DNA replication, cell division, regulatory proteins, cell walls, sugars, virulence, fatty acids, mRNAs, tRNAs, rRNAs, ribosomal proteins, proteins involved in protein synthesis, phospholipids, periplasmic proteins, secretory proteins, flagellar proteins, transport proteins, amino acids, lipopolysaccharides, purines, pyrimidines, pili, outer membrane proteins, nitrogen, antibiotic binding proteins and vitamins.

42. The composition of claim 40, wherein the oligonucleotide is capable of associating with a nucleic acid or protein in the bacterium such that it inhibits at least one of the group consisting of bacterial growth, reproduction, metabolism, synthesis of toxins, progress of infection and virulence.

43. The composition of claim 42, wherein the associating is hybridizing to an mRNA in the bacterium at or near the initiation codon, in the 5' untranslated region, in the 3' untranslated region, internal to the coding region or an intermediate region of the mRNA.

44. The composition of claim 42, wherein the associating is hybridizing to DNA in the bacterium.

45. The composition of claim 44, wherein the hybridizing forms a triplex structure.

46. The composition of claim 42, wherein the associating is binding with a protein in the bacterium.

47. The composition of claim 40, wherein the oligonucleotide hybridizes to any one of the operons listed in Table 1.

48. The composition of claim 40, wherein the oligonucleotide hybridizes to any one of the genes listed in Table 1.

49. The composition of claim 40, wherein the oligonucleotide comprises a sequence drawn from SEQ ID NOS. 1-176 of the Sequence Listing or a functional equivalent thereof.

50. The composition of claim 40, wherein the oligonucleotide has been purified by a method comprising at least one method from the group consisting of diafiltration, gel filtration, high performance liquid chromatography, fast performance liquid chromatography, alcohol precipitations or alcohol extractions followed by ethanol or chloroform extractions.

51. The composition of claim 40, wherein the oligonucleotide was purified by gel filtration.

52. The composition of claim 40, wherein the oligonucleotide is capable of inhibiting growth of the bacterium in an MIC assay.

53. The composition of claim 40, wherein the oligonucleotide has been modified in at least one base, sugar or internucleotide linkage so as to increase nuclease resistance, stability, specificity or uptake by bacteria of the oligonucleotide.

54. The composition of claim 40, wherein the oligonucleotide is selected from at least one of the group consisting of:

a) partially or fully substituted phosphorothioate oligonucleotides or analogues thereof;

b) partially or fully substituted alkyl phosphonate oligonucleotides or analogues thereof;

c) partially or fully substituted phosphate ester oligonucleotides or analogues thereof;

d) partially or fully substituted phosphoramidate oligonucleotides or analogues thereof;

e) partially or fully substituted 2' modified RNA oligonucleotides or analogues thereof;

f) partially or fully substituted morpholino oligonucleotides or analogues thereof;

g) partially or fully substituted peptide nucleic acid oligonucleotides or analogues thereof;

h) partially or fully substituted dithioate oligonucleotides or analogues thereof;

i) partially or fully substituted 5' thio oligonucleotides or analogues thereof;

j) partially or fully substituted propyne oligonucleotides or analogues thereof;

k) chimerics of any combination of the above; and

l) any chemical modifications of the oligonucleotide which leave the oligonucleotide capable of specifically binding the nucleic acid or protein.

55. The composition of claim 40, wherein the bacterium is gram positive.

56. The composition of claim 40, wherein the bacterium is gram negative.

57. The composition of claim 40, wherein the bacterium is acid fast.

58. The composition of claim 40, wherein the bacterium is a member of a genus selected from the group consisting of *Aerococcus, Listeria, Streptomyces, Actinomadura, Lactobacillus, Eubacterium, Arachnia, Mycobacterium, Peptostreptococcus, Staphylococcus, Corynebacterium, Erysipelothrix, Dermatophilus, Rhodococcus, Bifodobacterium, Lactobacillus, Streptococcus, Bacillus, Peptococcus, Micrococcus, Kurthia, Nocardia, Nocardiopsis, Rothia, Propionibacterium, Actinomyces, Enterococcus, Pneumococcus,* and *Clostridia*.

59. The composition of claim 40, wherein the bacterium is a member of the genus *Staphylococcus*.

60. The composition of claim 40, wherein the bacterium is *Staphylococcus aureus*.

61. The composition of claim 40, wherein the bacterium is a member of the genus *Pseudomonas*.

62. The composition of claim 40, wherein the bacterium is a member of the genus *Klebsiella*.

63. The composition of claim 40, wherein the bacterium is a member of the genus *Yersinia*.

64. The composition of claim 40, wherein the bacterium is a member of the genus *Neisseria*.

65. The composition of claim 40, wherein the bacterium is a member of the genus *Serratia*.

66. The composition of claim 40, wherein the bacterium is a member of the genus *Streptococcus*.

67. The composition of claim 66, wherein the bacterium is *Streptococcus pyogenes*.

68. The composition of claim 66, wherein the bacterium is *Streptococcus pneumoniae*.

69. The composition of claim 40, wherein the bacterium is a member of the genus *Shigella*.

70. The composition of claim 40, wherein the bacterium is a member of the genus *Haemophilus*.

71. The composition of claim 40, wherein the bacterium is a member of the genus *Mycobacterium*.

72. The composition of claim 40, wherein the bacterium is a member of the genus *Helicobacter*.

73. The composition of claim 40, wherein the bacterium is a member of the genus *Enterococcus*.

74. The composition of claim 40, wherein the bacterium is a member of the genus *Vibrio*.

75. The composition of claim 40, wherein the bacterium is a member of the genus *Salmonella*.

76. The composition of claim 40, wherein the bacterium is *Escherichia coli*.

77. The composition of claim 40, wherein the bacterium is *Pneumococcus*.

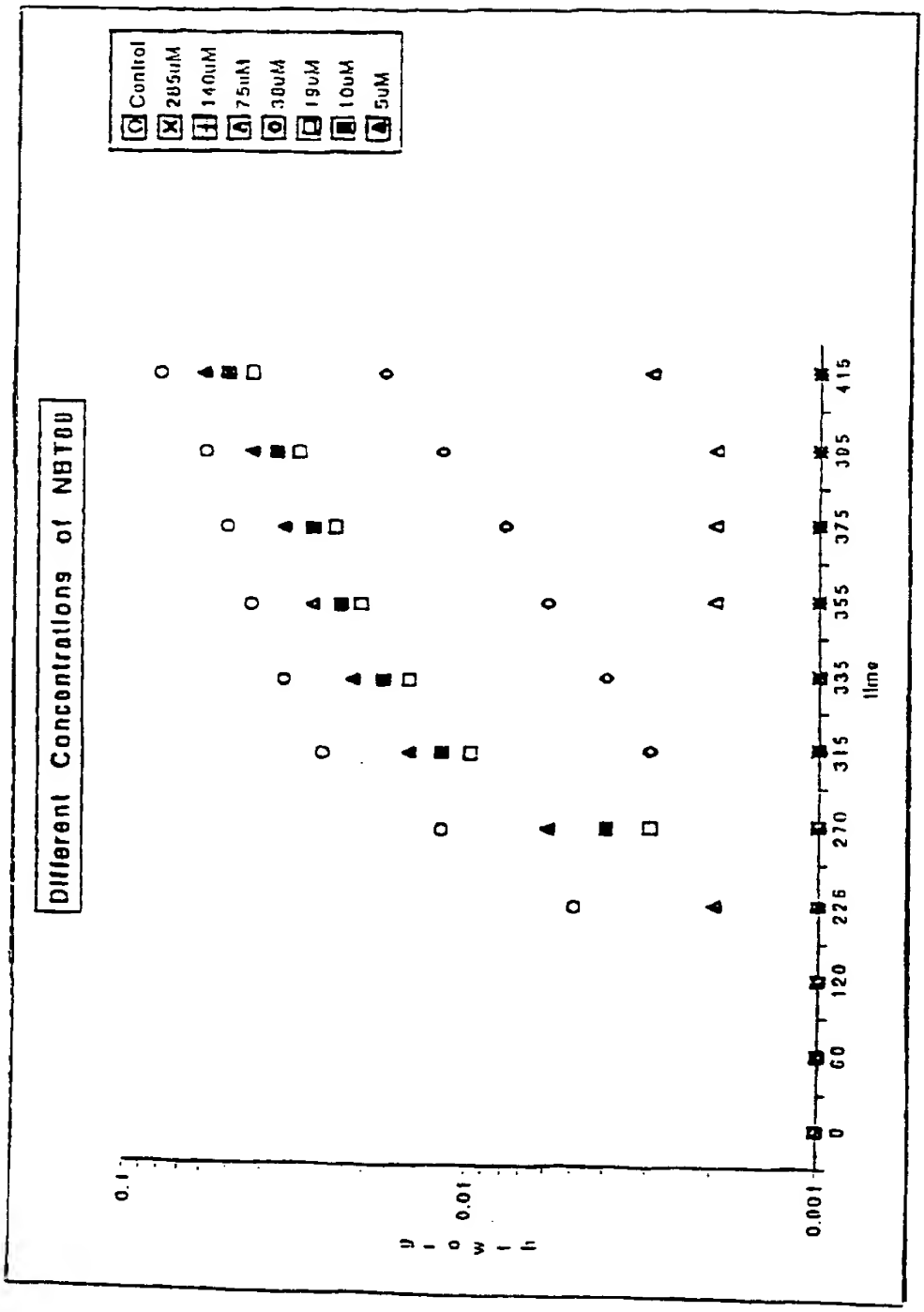
78. A compound, comprising:

a) an antibiotic; and

b) a substantially nuclease resistant oligonucleotide having about 8 to about 80 nucleotides and targeted to a nucleic acid or protein in a bacterium,

wherein said antibiotic is covalently linked to said oligonucleotide.

FIGURE 1


Different Concentrations of NBTDD
Control
285uM
140uM
75uM
38uM
19uM
10uM
5uM
0.1
0.01
0.001
0
60
120
225
270
315
335
355
375
395
415
time

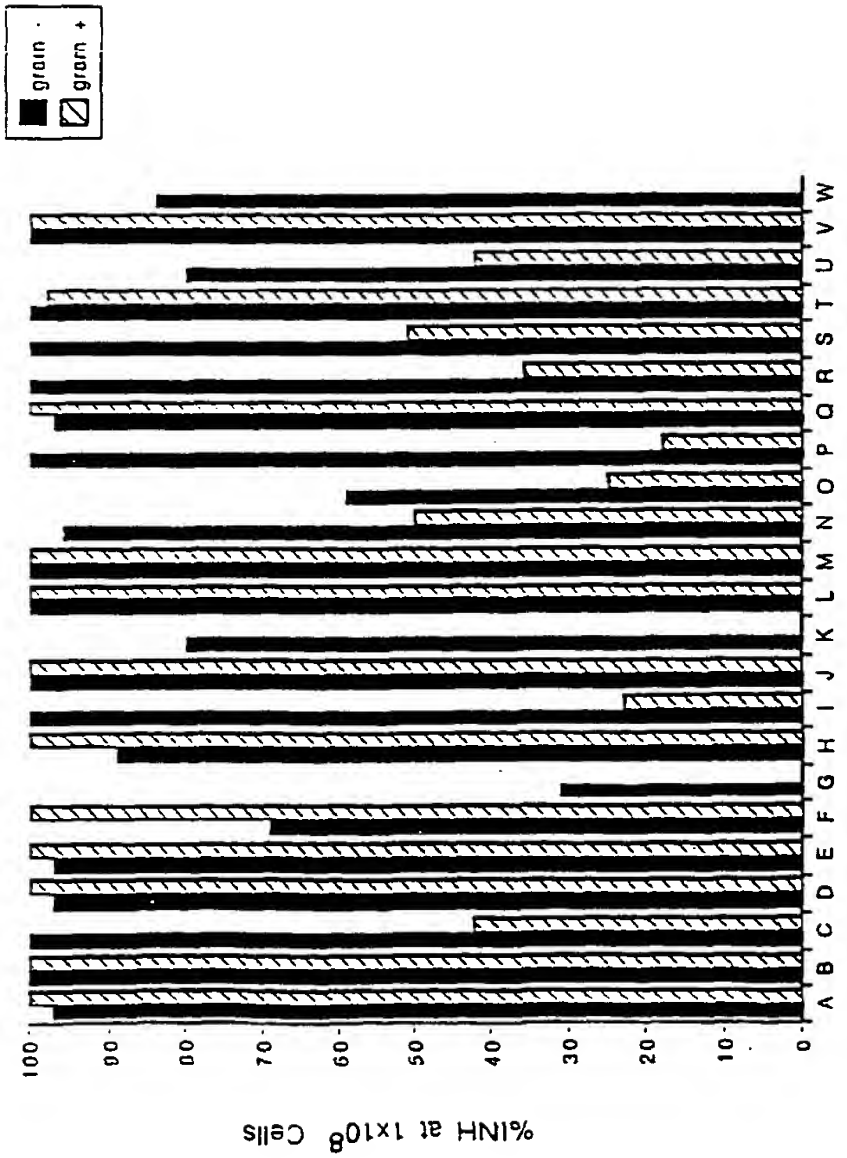
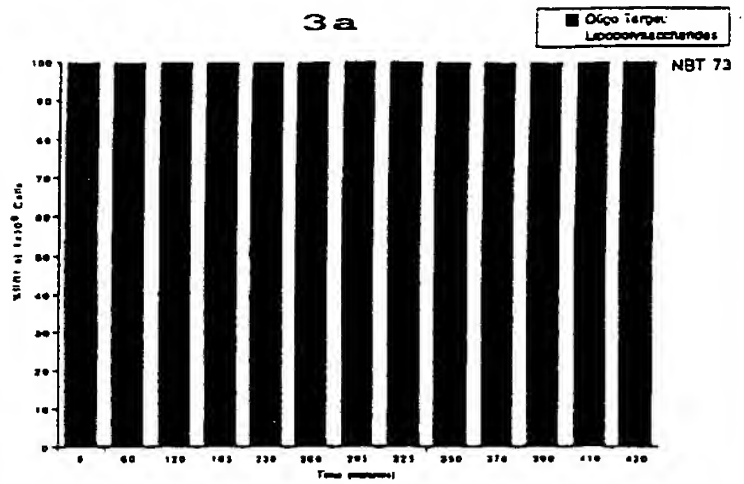

gram -
gram +
%INH at 1x10⁸ Cells
100
90
80
70
60
50
40
30
20
10
0
A B C D E F G H I J K L M N O P Q R S T U V W

FIGURE 2

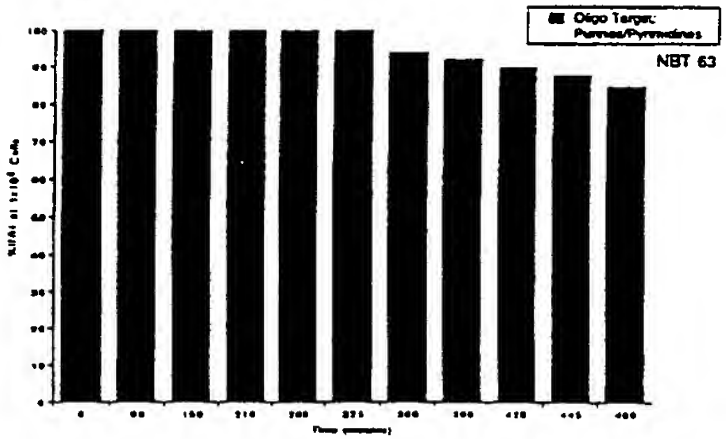
Growth Inhibition of Bacterial Strains with Oligos

Salmonella

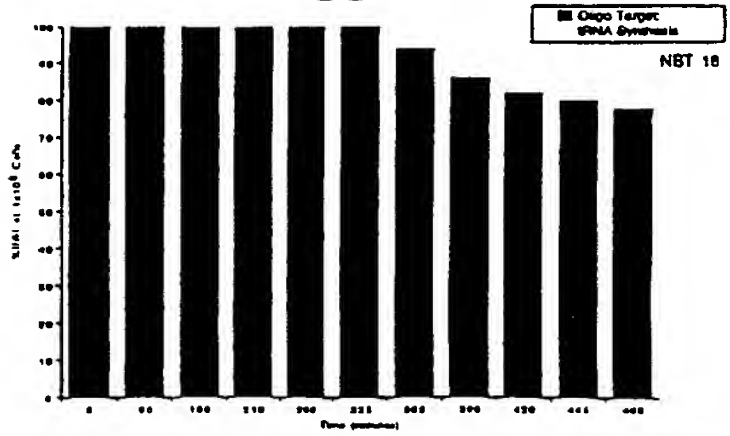
3a



3b



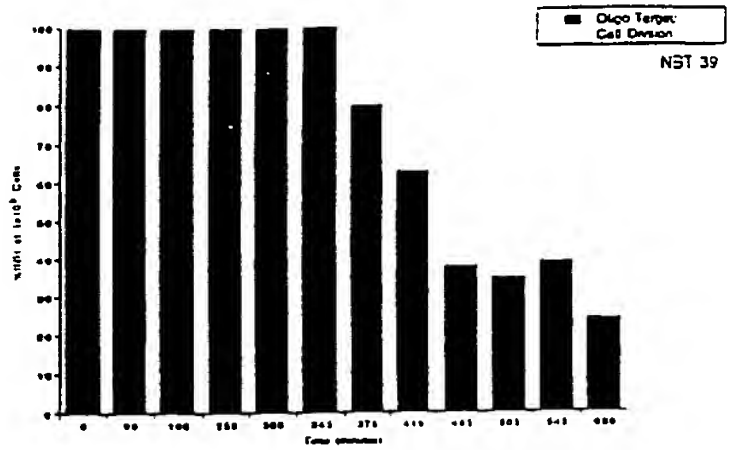
3c



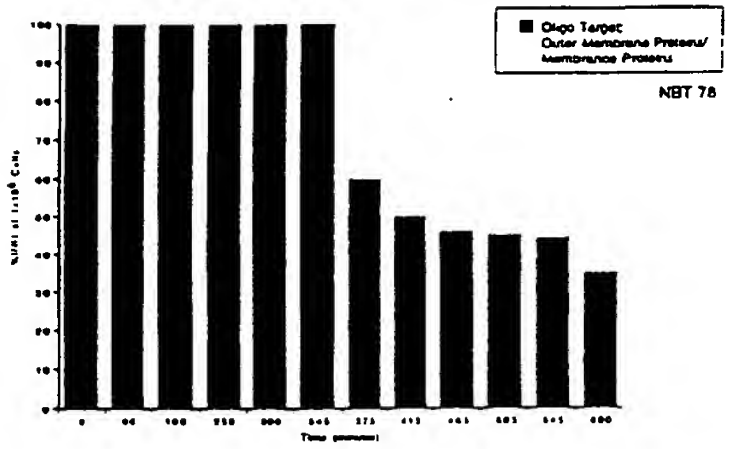
Growth Inhibition of Bacterial Strains with Oligos

Pseudomonas

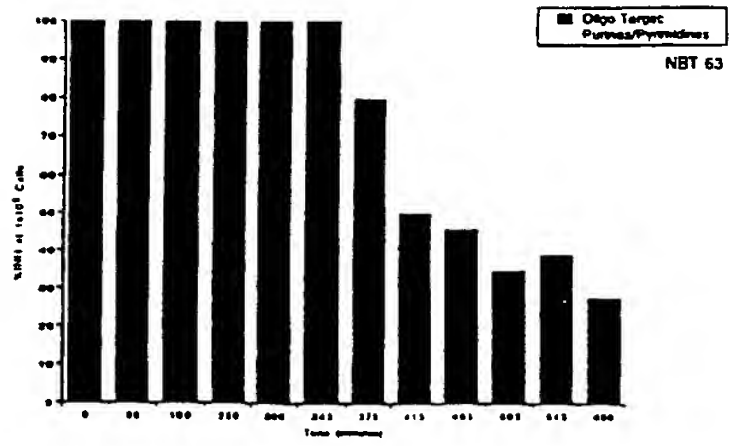
4a



4b



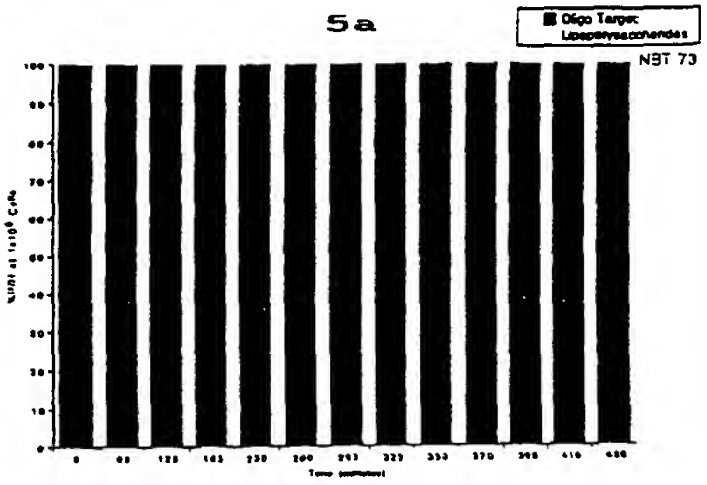
4c



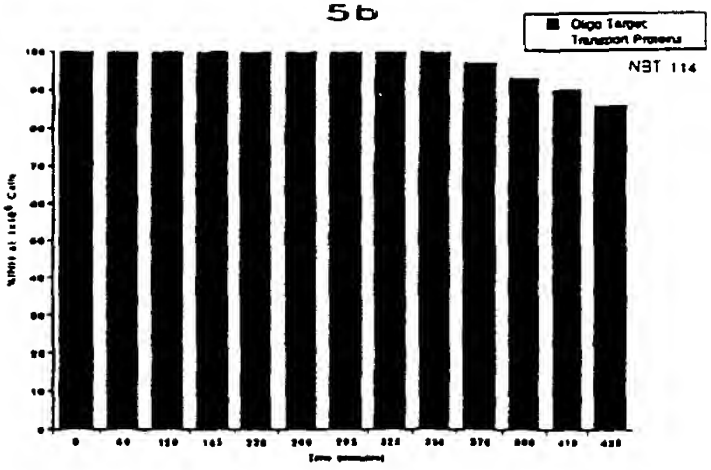
Growth Inhibition of Bacterial Strains with Oligos

Klebsiella

5a



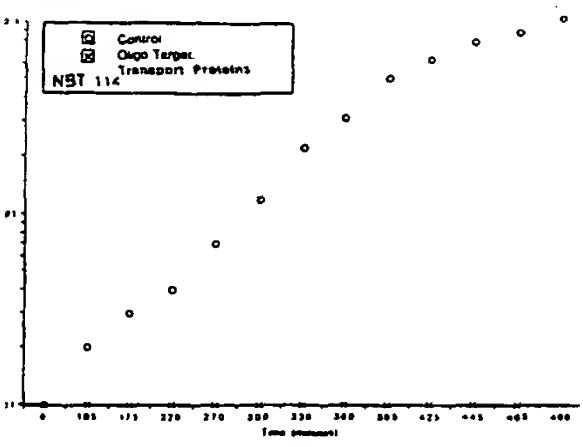
5b



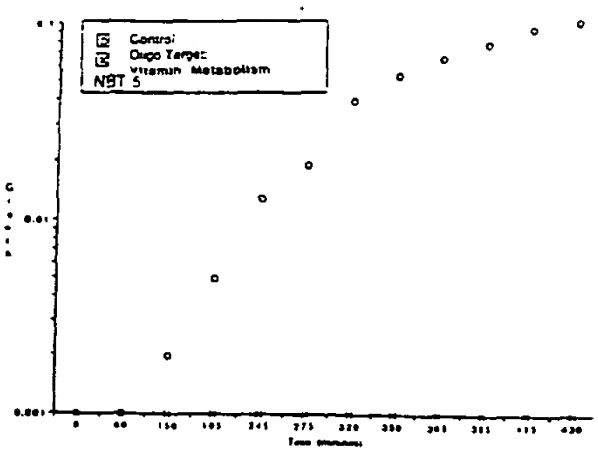
Growt. of E. coli 35218 (multiple-drug ..esistance) in the Presence of Oligos

6a

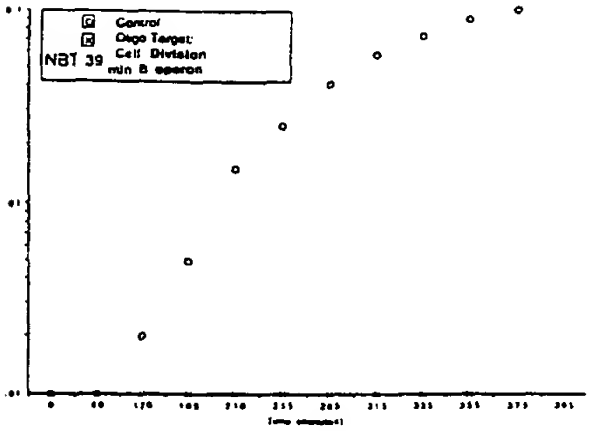
Growth measured at 600 nanometers



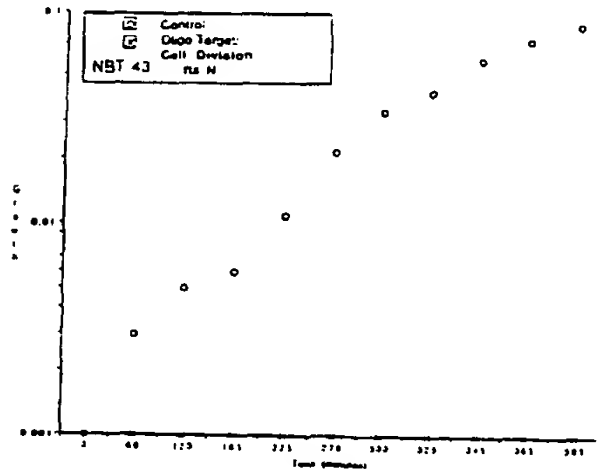
6b



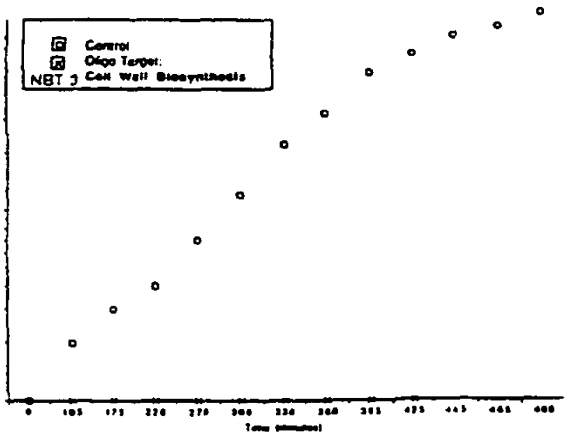
6c



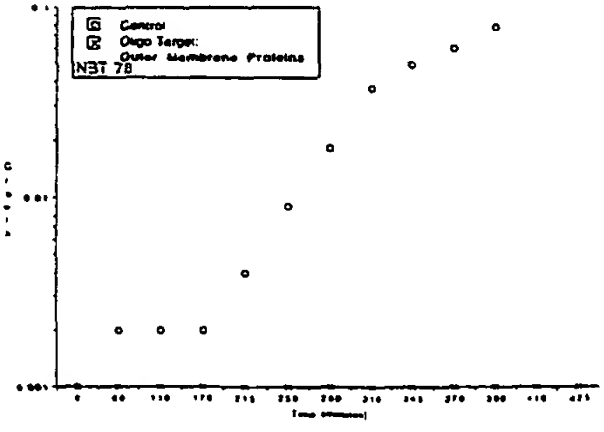
6d



6e



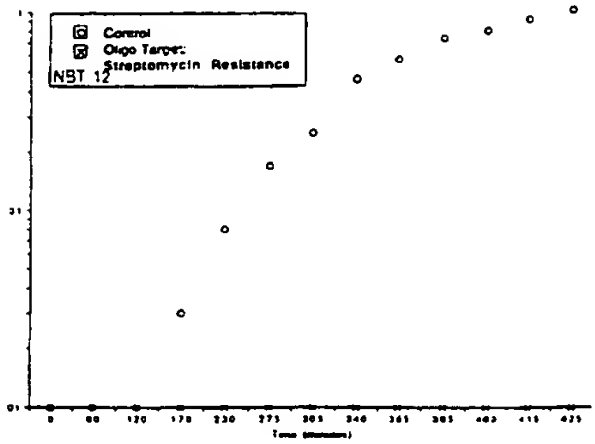
6f



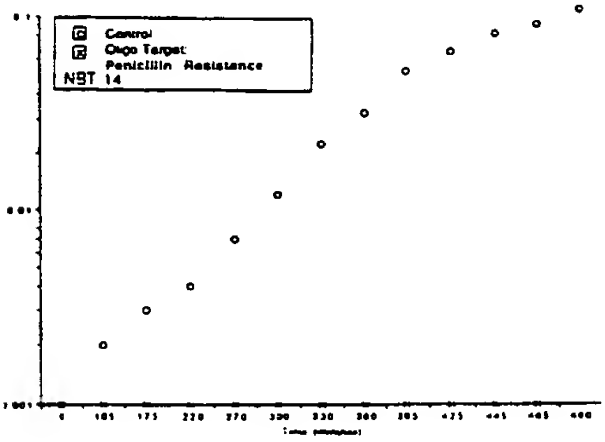
Growth of E. coli 35218 (multiple drug resistance) in the Presence of Oligos

6g

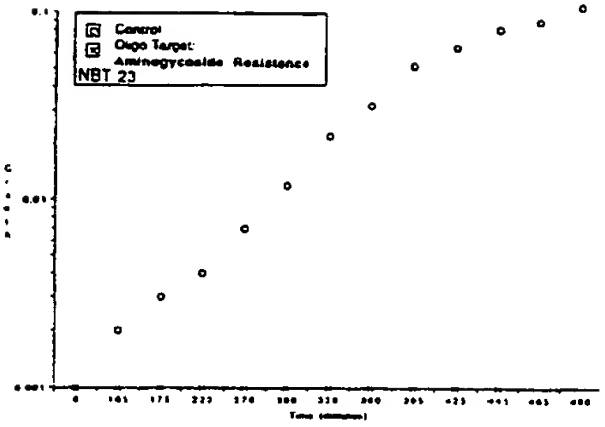
Growth measured at 600 nanometers



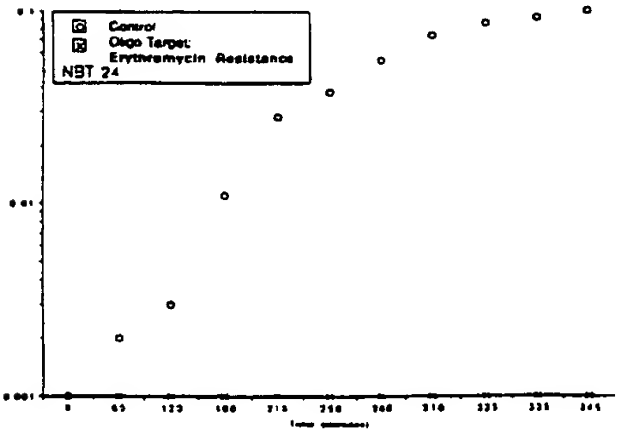
6h



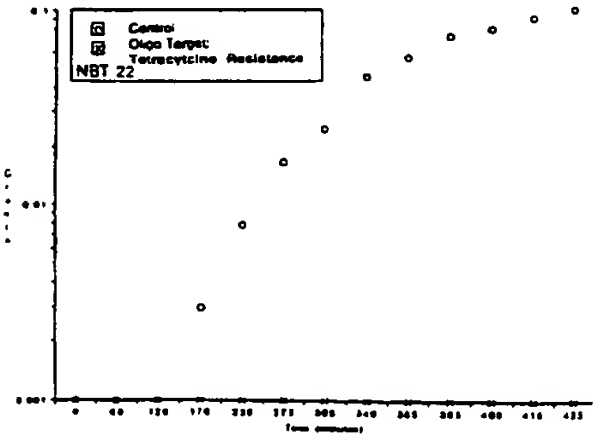
6i



6j



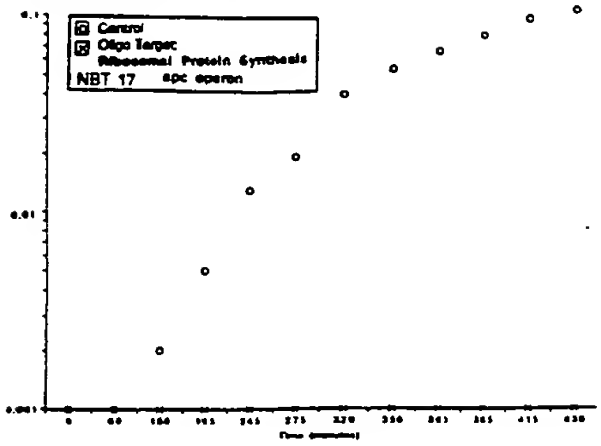
6k



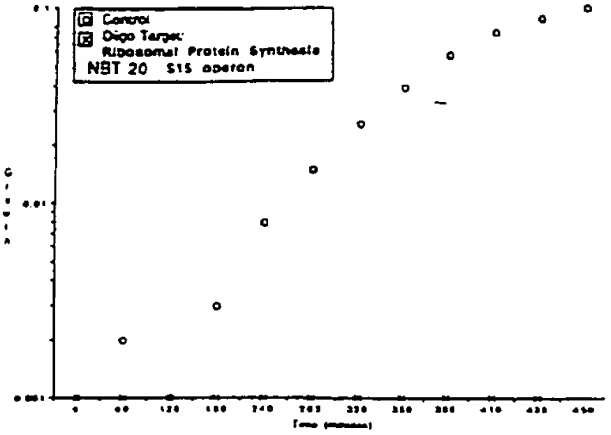
Growth of E. coli 35218 (multiple drug resistance) in the Presence of Oligos

6l

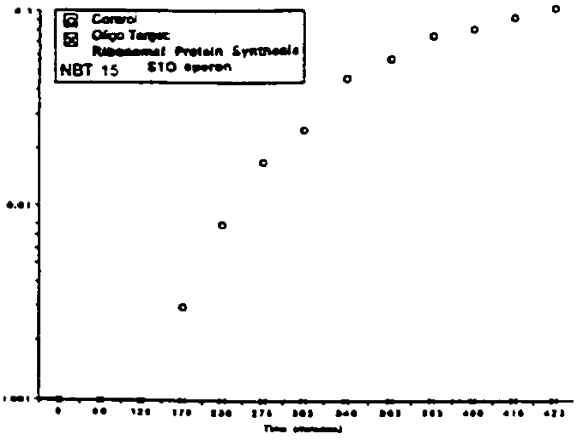
Growth measured at 600 nanometers



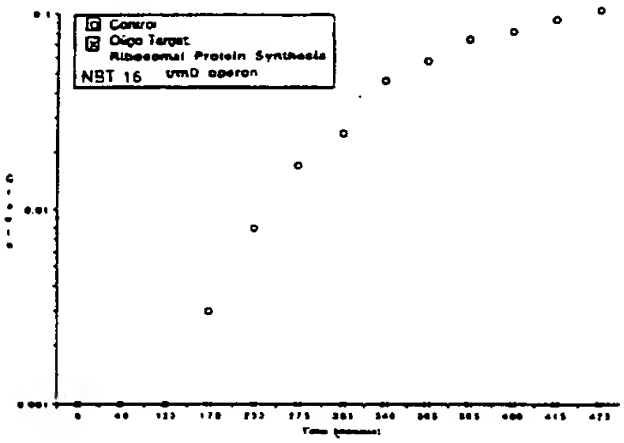
6m



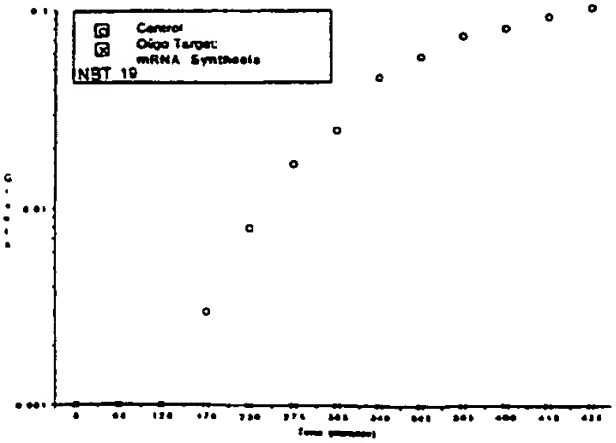
6n



6o



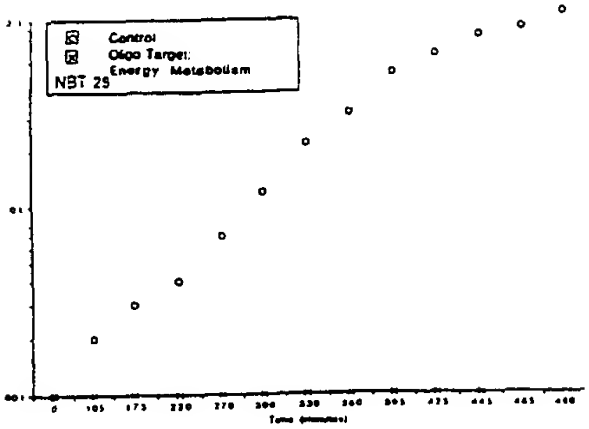
6p



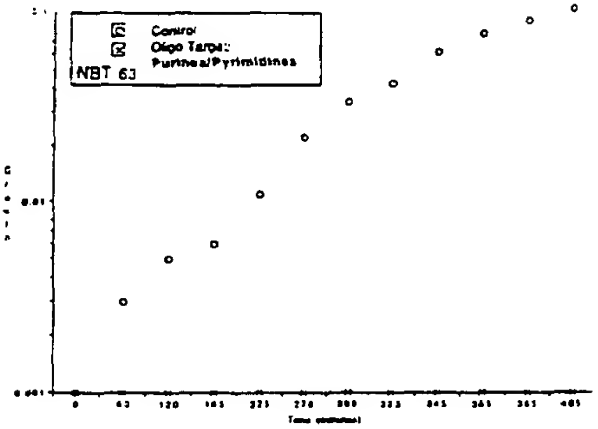
Growth of E. coli 35218 (multiple drug resistance) in the Presence of Oligos

Growth measured at 600 nanometers

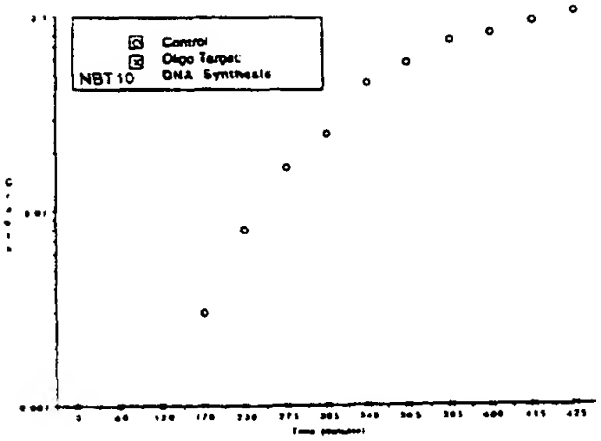
6q



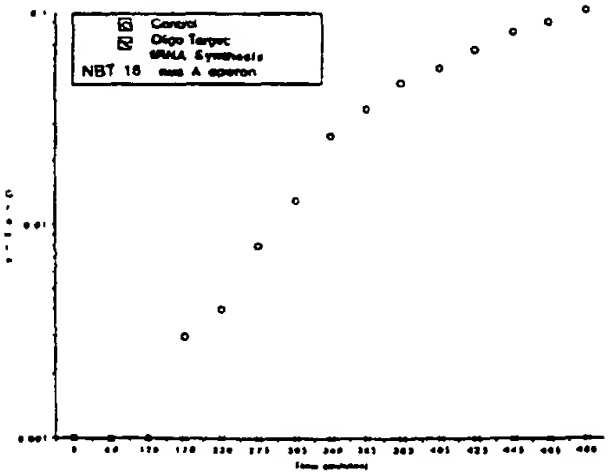
6r



6s



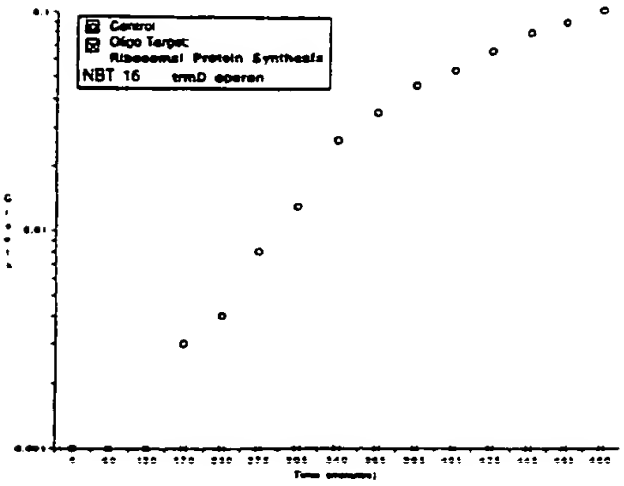
6t



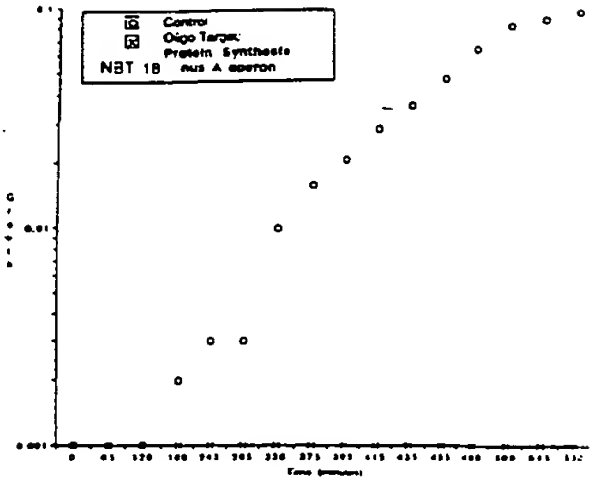
Growth Inhibition of Staph 13301 (penicillin resistant) in the Presence of Oligos

Growth measured at 600 nanometers

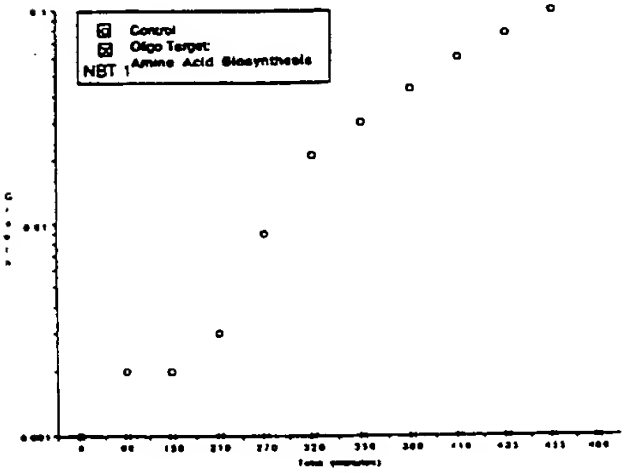
7a



7b



7c



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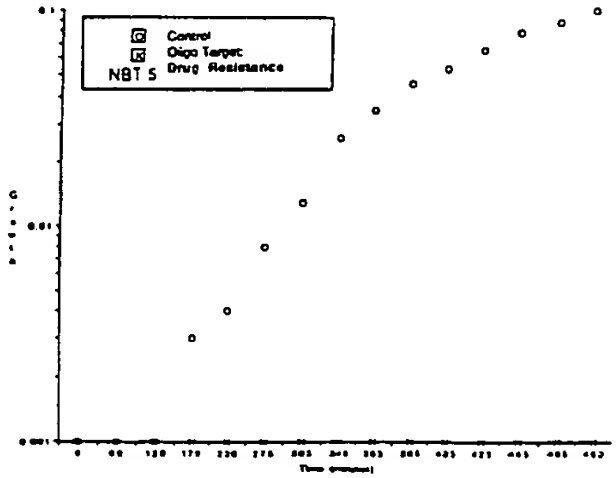

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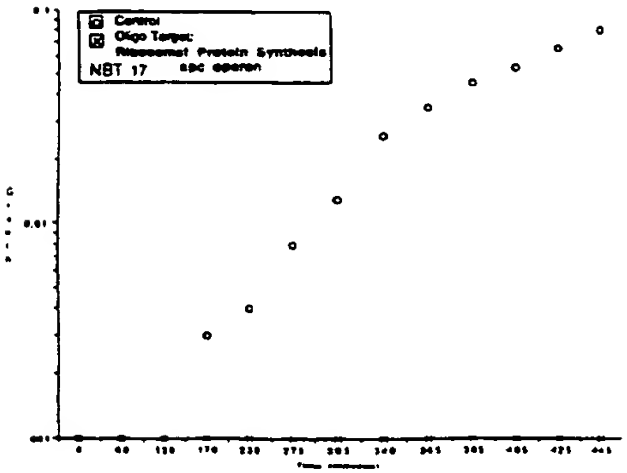
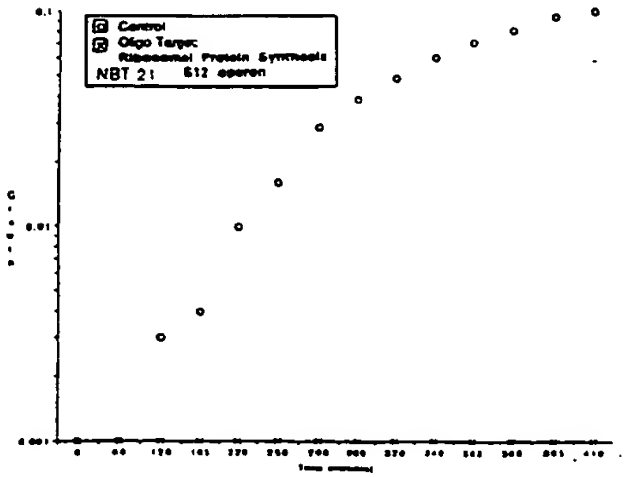


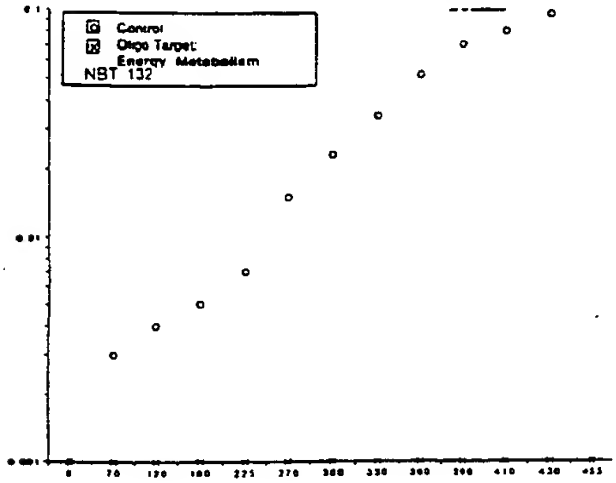
7f



Growth Inhibition of Staph 13301 (penicillin resistant) in the Presence of Oligos

owth measured at 600 nanometers

7g



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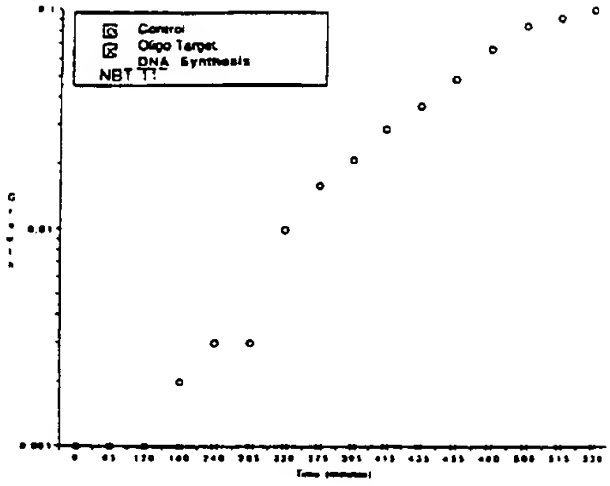

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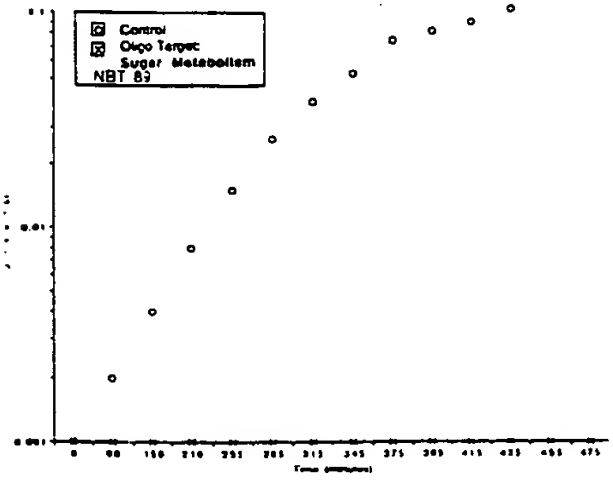

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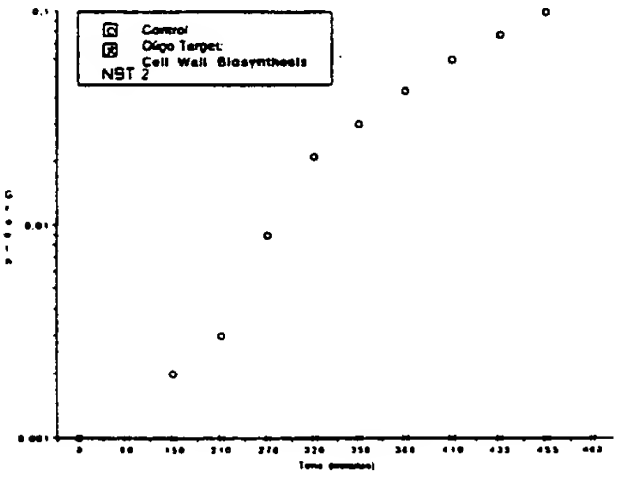


Animal Data

A) Lister Model

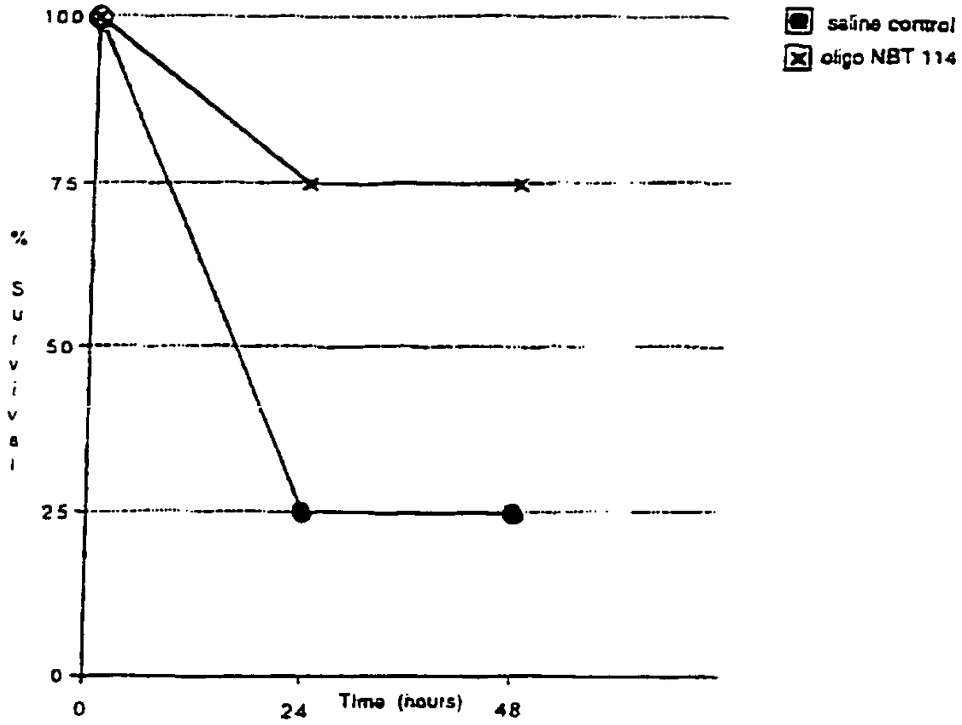


FIGURE 8

In Vivo Efficacy

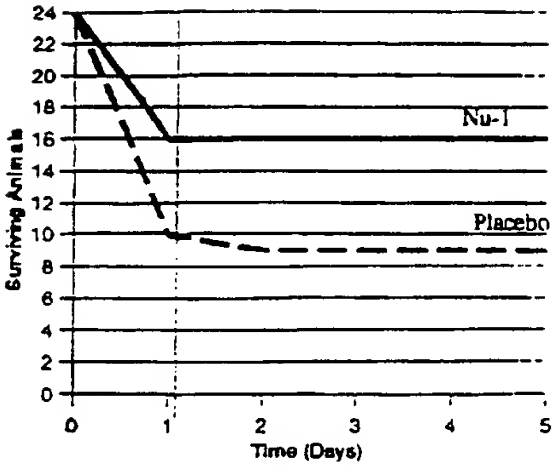


FIGURE 9

Standard Overnight MIC Assay- *Staph. aureus* 3 Day Time Course

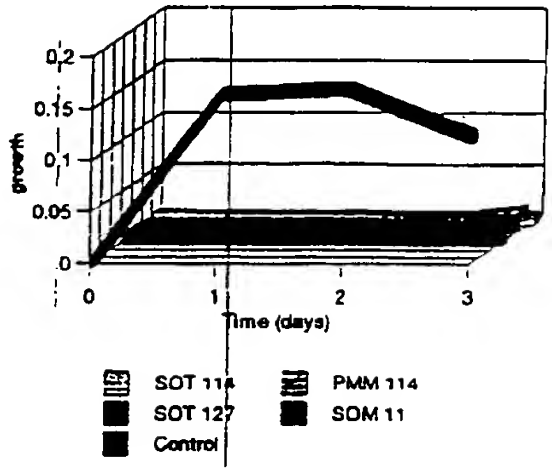


FIGURE 10a

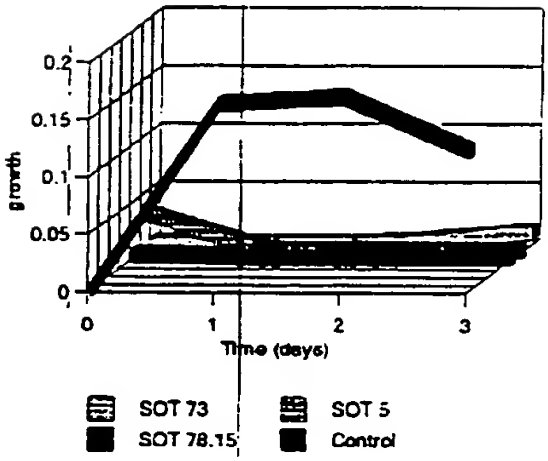


FIGURE 10b

Standard Overnight MIC Assay
Serratia liquefaciens

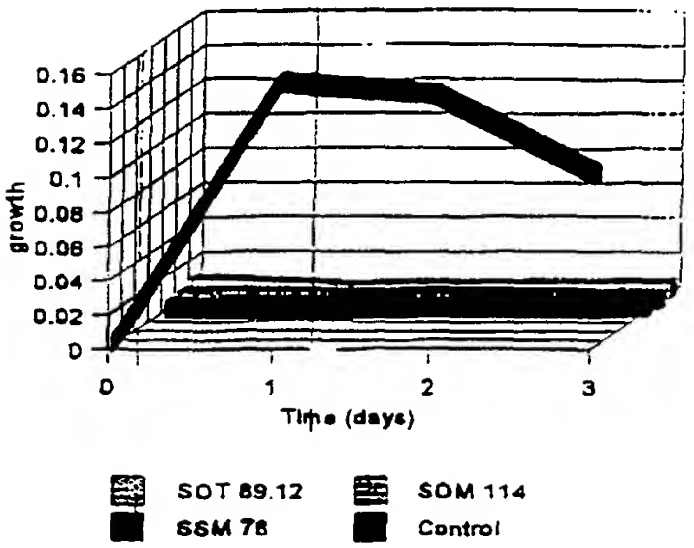


FIGURE 11a

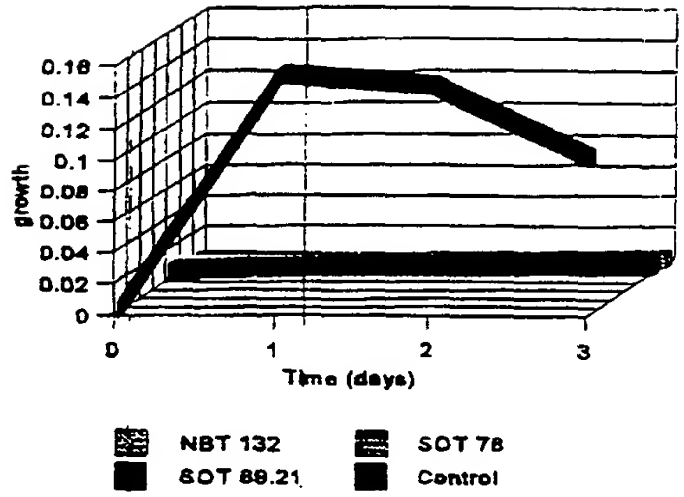
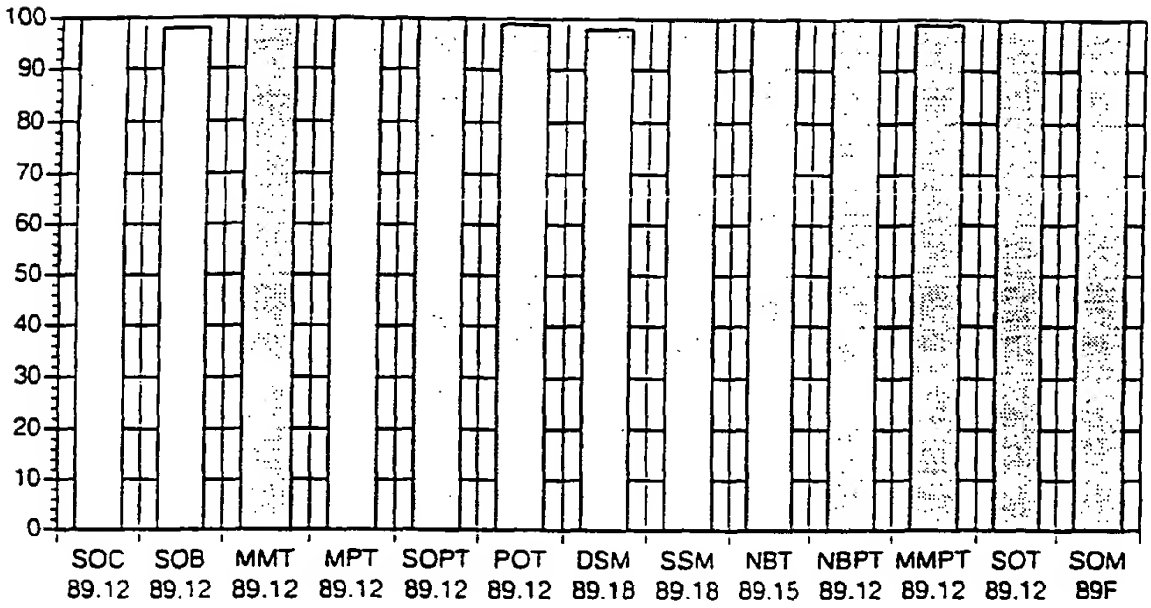


FIGURE 11b

FIGURE 12

Standard MIC Assay
Staph. aureus

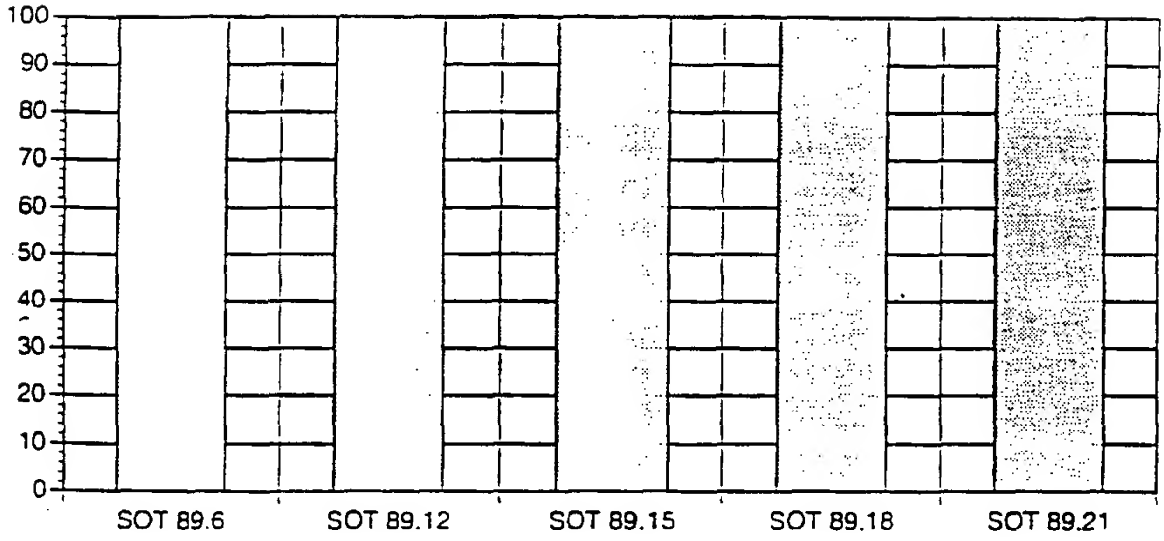


SOC - 5' - 6 Ds 6Mo - Cholesteryl - 3'
SOB - 5' - 6 Ds 6Mo - Biotin - 3'
MMT - 5' - 12 Mo Invert T - 3'
MPT - 5' - 10 Mo 2Mp Invert T - 3'
SOPT - 5' - 6 Ds 4 Mo 2Mp Invert T - 3'
POT - 5' - 12 Po (Invert T) - 3'
DSM - 5' - 8 Ds 10 Ms 1 Do - 3'
SSM - 5' - 18 Ms 1 Do - 3'
NBT - 5' - 14 Ds Do - 3'
NBPT - 5' - 10 Ds 2Mp Invert T - 3'
MMPT - 5' - 10 Mo 2 Mp Invert T - 3'
SOT - 5' - 6 Ds 6Mo Invert T - 3'
SOM-F - 5' - 1 Ms 4Ds 12 Mo 3 Ms 1 Do - 3'

Different constructs that work well in inhibition of bacterial growth.

FIGURE 13

Standard MIC Assay
Staph. aureus



SOT 89.6-6mer
SOT 89.12-12 mer
SOT 89.15-15 mer
SOT 89.18-18 mer
SOT 89.21-21 mer

Oligos of different lengths work well in inhibition of bacterial growth.

Comparison of Oligo 114 and Ampicillin

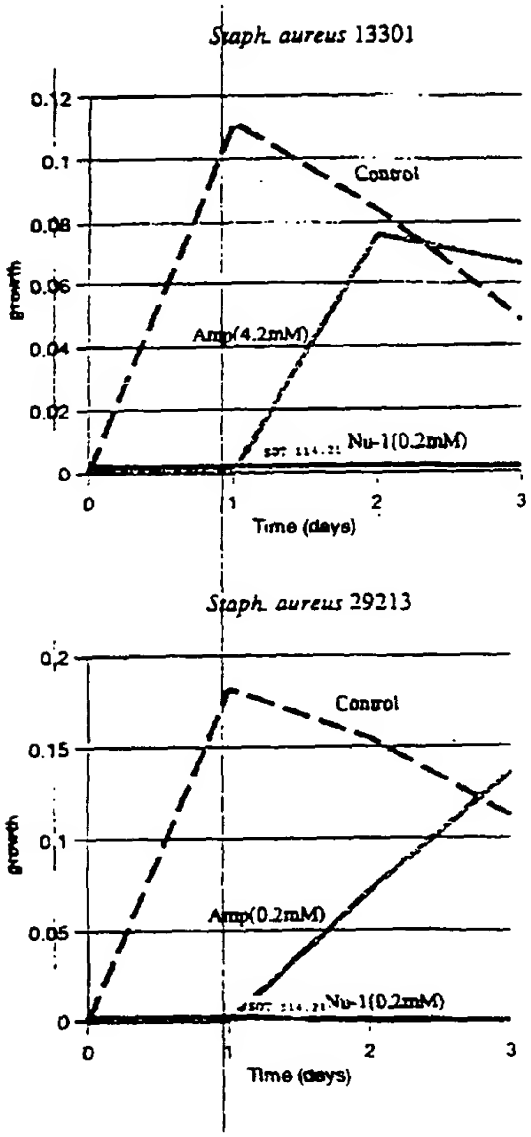


FIGURE 14

Pseudomonas aeroginosa 10145

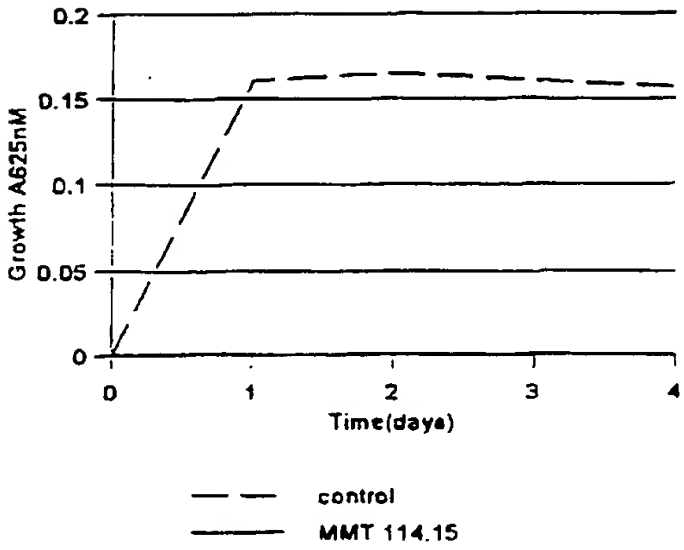


FIGURE 15

Streptococcus pyogenes 14289

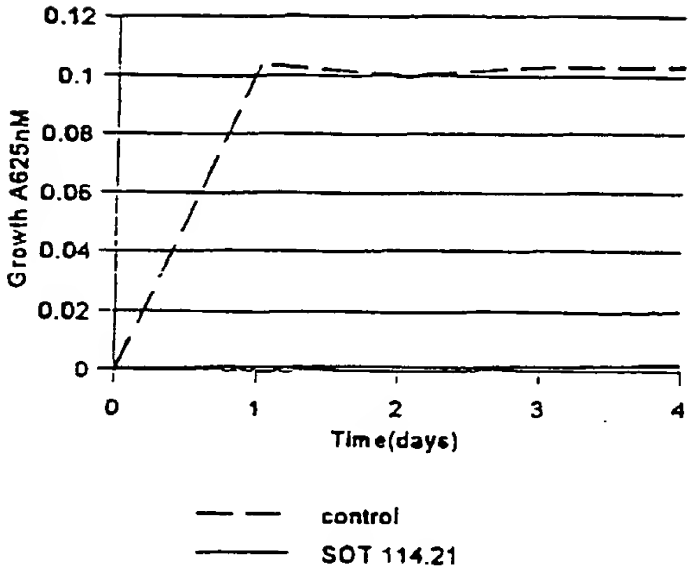


FIGURE 16

INTERNATIONAL SEARCH REPORT

Intern. nal Application No
PCT/US 97/12961

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H21/00 A61K31/70 C12N15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07H A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 294 533 A (LUPSKI JAMES R ET AL) 15 March 1994 cited in the application see the whole document ---	1-77
P,X	WO 96 29399 A (SOD CONSEILS RECH APPLIC ;PIROTZKY EDUARDO (FR); COLOTE SOUDHIR (F) 26 September 1996 see the whole document ---	1-77
A	L. A. CHRISEY ET AL.: "Internalization of Oligodeoxyribonucleotides by Vibrio parahaemolyticus" ANTISENSE RES. DEV., vol. 3, 1993, pages 367-381, XP002045887 cited in the application --- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
6 November 1997	16. 12 97

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A	M.A. RAHMAN ET AL.: "Antibacterial Activity and Inhibition of Protein Synthesis in Escherichia coli by Antisense DNA Analogs" ANTISENSE RES. DEV., vol. 1, 1991, pages 319-27, XP002045888 cited in the application	
A	K. JAYARAMAN ET AL.: "Selective Inhibition of Escherichia coli Protein Synthesis and Growth by Nonionic Oligonucleotides Complementary to the 3' End of 16S rRNA" PROC. NATL. ACAD. SCI. USA, vol. 78, 1981, pages 1537-41, XP002045889 cited in the application	

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Information on patent family members

Internat. ..al Application No
PCT/US 97/12961

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5294533 A	15-03-94	AU 645339 B	13-01-94
		AU 8263491 A	27-02-92
		CA 2048450 A	24-02-92
		EP 0472434 A	26-02-92
		JP 6303977 A	01-11-94
		AT 137806 T	15-05-96
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		DE 68926455 D	13-06-96
		DE 68926455 T	31-10-96
		EP 0424473 A	02-05-91
		JP 3505672 T	12-12-91
		WO 9000624 A	25-01-90
WO 9629399 A	26-09-96	AU 5149796 A	08-10-96

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(30) Priority Data:
08/685,575 24 July 1996 (24.07.96) US

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(74) Agents: FRIEBEL, Thomas, E. et al.; Pennie & Edmonds LLP, 1155 Avenue of the Americas, New York, NY 10036 (US).

(81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GE, GH, HU, IL, IS, JP, KG, KP, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
With amended claims.

Date of publication of the amended claims:
2 April 1998 (02.04.98)

(54) Title: ANTISENSE OLIGONUCLEOTIDES AS ANTIBACTERIAL AGENTS

(57) Abstract

A novel method is provided that teaches the therapeutic use of nuclease resistant oligonucleotides for treating animals having an infection caused by a pathogenic bacterium. The method involves the integration of (1) methods for selecting the correct oligonucleotide, (2) synthesis and purification of nuclease resistant oligonucleotides, and (3) methods for *in vitro* analysis of potential antimicrobial oligonucleotides. The described oligonucleotides may comprise modified backbones, sugar residues, bases, or mixtures and have been subject to purification resulting in oligonucleotides that are capable of inhibiting the growth of a broad spectrum of clinically relevant bacterial species.

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AMENDED CLAIMS

[received by the International Bureau on 12 February 1998 (12.02.98); original claims 1-78 replaced by new claims 1-21 (2 pages)]

1. The use of a substantially nuclease resistant oligonucleotide having about 8 to about 80 nucleotides in the preparation of a medication for the treatment of infection by pathogenic bacteria.

2. The use of claim 1 wherein said bacteria are gram positive.

3. The use of claim 2 wherein said bacteria is selected from the group consisting of: *Aerococcus, Listeria, Streptomyces, Actinomadura, Lactobacillus, Eubacterium, Arachnia, Mycobacterium, Peptostreptococcus, Corynebacterium, Erysipelothrix, Dermatophilus, Rhodococcus, Bifodobacterium, Lactobacillus, Bacillus, Peptococcus, Micrococcus, Kurthia, Nocardia, Nocardiopsis, Rothia, Propionibacterium, Actinomyces, Pneumococcus,* and *Clostridia*.

4. The use of claim 2, wherein the bacterium is a member of the genus *Staphylococcus*.

5. The use of claim 4, wherein the bacterium is *Staphylococcus aureus*.

6. The use of claim 2, wherein the bacterium is a member of the genus *Streptococcus*.

7. The use of claim 6, wherein the bacterium is *Streptococcus pyogenes*.

8. The use of claim 6, wherein the bacterium is *Streptococcus pneumoniae*.

9. The use of claim 2, wherein the bacterium is a member of the genus *Enterococcus*.

10. The use of claim 1 wherein said bacteria are gram negative.

11. The use of claim 10, wherein the bacterium is a member of the genus *Pseudomonas*.

12. The use of claim 10, wherein the bacterium is a member of the genus *Klebsiella*.

13. The use of claim 10, wherein the bacterium is a member of the genus *Yersinia*.

14. The use of claim 10, wherein the bacterium is a member of the genus *Neisseria*.

15. The use of claim 10, wherein the bacterium is a member of the genus *Serratia*.

16. The use of claim 10, wherein the bacterium is a member of the genus *Shigella*.

17. The use of claim 10, wherein the bacterium is a member of the genus *Haemophilus*.

18. The use of claim 10, wherein the bacterium is a member of the genus *Mycobacterium*.

19. The use of claim 10, wherein the bacterium is a member of the genus *Vibrio*.

20. The use of claim 10, wherein the bacterium is a member of the genus *Salmonella*.

21. The use of claim 10, wherein the bacterium is *Escherichia coli*.